

Derivation of functional retinal endothelial cells from human pluripotent stem cells for therapeutics and modeling

Corresponding Author: Professor Sharon Gerecht

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Report for the authors (Required))

Summary

In this manuscript, Lin et al develop a protocol to differentiate retina-like ECs from hiPSCs and characterize them transcriptionally, and functionally in vitro and in vivo. They achieve this by addition of Norrin and vitronectin to the differentiation and 2 rounds of sorting for CD31 and Fz4 to induce iREC 'specification'. "generic" EC –not treated with Norrin and vitronectin–were used as control for retina-specific assessments. Authors used 2 different hiPSC lines and compared iRECs to primary retinal ECs. They tested the effects of HG media and hypoxia on iREC junctions and TEER and used a mouse model of oxygen-induced retinopathy to investigate iREC incorporation into the developing eye vasculature where PBS injection to the contralateral eye was used as control. Finally, they used microphysiological systems to seed iREC and pericytes to investigate inner blood-retinal barrier properties in vitro.

Degree of advance

The field of endothelial organotypic identity and plasticity has recently attracted considerable attention. Seminal questions related to EC organotypic identity in engineered tissues and regenerative medicine are unanswered. Key questions relate to whether ECs can adopt the organ-specific characteristics of their implantation site or if organ-specific ECs are required for tissue/organ function in transplantation. Papers addressing these seminal questions should draw significant attention and be highly cited. While Lin et al tangentially address some aspects of this exciting area of research, the lack of appropriate controls (generic ECs) for functional readouts in both in vivo and in vitro models have dampened the enthusiasm for the findings.

There is certainly a methodological advance: the development of an EC differentiation protocol to generate retina-like ECs. However, not enough evidence was given, in its present form, to provide significant fundamental, mechanistic, or performance-wise advances – which would increase significantly the impact of this paper.

Implication of the findings

As stated by the authors, 'there is an increasing need to develop tissue-specific ECs for more precise disease modeling and effective tissue regeneration and repair'. However, there was no demonstration in this manuscript that the retina-like ECs more precisely model the retina EC behavior or show tissue-specific function in vitro and in vivo when compared to generic ECs.

While generating retina-like ECs and studying their general characteristics certainly has merit, the greatest advancement would be to provide evidence that it is advantageous to use tissue-specific ECs (in this case iRECs) in vitro (as even native primary tissue-specific cells lose phenotype in culture) and in vivo compared to 'generic' ECs or cells from other tissues. Given that the prevailing dogma is that ECs are plastic and can adapt to the local environment, the significant higher cost and time needed to differentiate tissue-specific ECs needs to be justified. However, authors failed to provide convincing evidence of the advantages of using iREC, as most assays performed lacked a direct comparison to generic ECs or ECs of different origins. Without this, the advancement to the field is modest and future adoption by others will be limited. Towards that goal, authors should provide appropriate controls for functional assays.

Major criticism

1- Lack of 'generic' EC controls in both in vitro and cell transplantation assays limits the conclusions

2- It is unclear why the authors elected to test the vessel formation in MPS and not provide assessment of barrier function

under fluid flow – which, as they point out themselves “enabling the study of tissue function” and this is the best application of MPS that is also relevant for their hypothesis. iRECs permeability coefficient should be provided and compared to iECs in the chip, under flow, to assess if they indeed enable improved barrier function. While TEER is a good method, it lacks functional assessment, which can be achieved in the chip (see Pham Lab on a chip 2017, Chen lam Chip 2023 etc.).

3- On TEER: what was the media used for the experiments? Methods describe that iECs were cultured in media with 50ng/ml of VEGF while iRECs in the same media but without VEGF – see excerpt below. Given that VEGF is well known for inducing EC permeability, differences in TEER could be due to the different culture media used. Results must be provided for assays performed with the same media for all cells.

“The purified CD31+ iECs were plated on a type-I collage-coated plate and expanded and maintained in ECGM supplemented with 10 μ M SB-431542, 50 ng/ml VEGF-A, and 10 μ M Y-27632. After 24 hours, the media was changed to remove Y-27632. After that, media was changed every other day.”

“iRECs were plated on a type-I collagen-coated plate and expanded and maintained in ECGM supplemented with 10 μ M RepSox, 10% platelet poor human serum (Millipore Sigma), and 10 μ M Y-27632. After 24 hours, the media was changed to remove Y-27632 and then media was changed every other day.”

4- There are not enough details in the methods to reproduce the findings, so methods need to be improved substantially. Cell seeding density at thawing and passaging and for assays need to be provided, how many cells were collected in 100ul of FACs buffer, etc.

5- It would be good to have a comparison between iREC and iECs for glut1 inhibition to show specificity

6- Fig 2 Cii, Eiii; S3a, b, Fig 5C are not really useful as it is difficult to draw conclusions from 1 image. For rigour, assessments must be provided as in Fig 2Dii.

7- Diabetic retinopathy conditions used in the manuscript are extreme: 30mM of glucose is extremely high and then there's the presence of 1% O₂ – for up to 72h. I am amazed any cells were still alive. Assessment of cell viability (e.g. live dead stain) would be useful to understand the extent of the DR treatment. Authors should also provide a better justification for using such an extreme model. Moreover, comparison with iECs should be done. Are iRECs better at modeling diabetic retinopathy? In which aspects? This would be extremely valuable for the field and highlight the advantages of using tissue-specific ECs.

8- Was the hypothesis that DR would induce loss of Claudin-2 junctions? Is that why only iRECs were used in this assay? Is this what has been reported in DR in vivo and that the authors have tried to recapitulate?

9- It is not clear if that for the DR TEER assessment the cells were seeded in the same media, allowed to attach and form a monolayer and then subjected to DR model as there seem to be differences, albeit small, already at day 0.

10- Instead of comparing to iPSC-derived brain ECs, it would be more relevant to perform an unbiased scoring of the iECs and iRECs against DEGs in native RECs (which can also be obtained from published data, e.g. Menon et al Nat. Communications 2019; Watson et al Mol Vis 2020 etc.) to demonstrate which would score higher, without picking and choosing genes.

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14- Does the inclusion of pericytes improve vessel formation, barrier function etc? This whole pericyte bit feels disconnected from the other parts of the manuscript. Perhaps using the model to study DR would be more relevant?

Minor criticism

Fig S1 caption reads: “and functional analysis” but there is no functional analysis reported. Panel C should also show generic ECs.

Fig S2 would benefit from quantitative analysis

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Providing only 1 image without quantification demonstrate lack of rigor.

Provide lower magnification images for Fig 5 Dii and assessment of network perfusion

missing or unclear details about statistics, protocols or materials

For quantifications (e.g. Fig 2Dii) authors need to provide details of what was assessed, were all points measured from the same assays or 8 different biological replicates (differentiations and different runs)? In other words, are these technical

replicates of real separate assays?

Reviewer #2

(Report for the authors (Required))

The reviewed paper aims to modify the existing differentiation protocol of endothelial cells (ECs) and pericytes (PCs) from iPSCs to mimic the retinal EC and PC phenotypes. Although the proposed project possesses high impact due to providing materials for otherwise hardly accessible cell types for disease modeling and therapeutic approaches, the current state of the project cannot yet be considered complete.

(1) The authors modified the existing protocol for electroporation-induced EC differentiation to drive additionally treating with Norrin and vitronectin to induce the retinal EC (iRECs). As a result the cell population iREC positive for CD31 and Fz4 was received after two rounds of cell sorting.

For the iREC induction protocol, I have the following questions:

Why was the electroporation protocol chosen as a base protocol? Did the authors try standard EC induction with growth factors and it didn't work or was the electroporation protocol the first choice? Electroporation has limitations for possible therapeutic usage: although a non-integrative method was used, it still increases the risk for genomic instability. Were there any functional assays done to prove the EC differentiation? Several previous attempts to receive brain ECs phenotype resulted instead in the differentiation of epithelial-like cells then ECs. Therefore it is essential to verify the EC identity of iRECs with functional assays, such as (1) an actively take-up acetylated LDL and (2) ICAM expression upon TNF- α treatment. These additional experiments would confirm that ECs preserved cell identity after Norrin and vitronectin induction.

(2) The authors used the expression of two cell markers, CD31 and Fz4, as hallmarks of retinal EC and PC specification. However, both iECs and pre-iRECs were shown to express CD31 and Fz4. iREC induction was performed by adding the Fz4 ligand, Norrin. The efficiency of iREC differentiation was assessed by the increase in the Fz4⁺ cell population. But isn't the most obvious explanation for this increase a positive feedback loop, where Norrin induces the expression of its receptor, Fz4? If so, the results may reflect Norrin's effect on ECs rather than the emergence of a distinct iREC population.

(3) Figure 1D demonstrates the differences in morphology and marker expression of iECs and iREC. The morphology of iECs appears healthier: cells are uniformly shaped and properly aligned with each other. The round and irregular shape of iRECs, on the other hand, reminds features of senescence or dysfunctional ECs.

(4) Figure S2 demonstrates the staining and morphology of HRECs. The cell density appears low and cell morphology is generally unhealthy. Authors should consider excluding these cells from the analysis since results received with unhealthy cells are unreliable.

(5) Figure S2C should prove the robustness of the protocol by using another hiPSC line to differentiate iRECs. However, the authors do not show the expression of ZO-1 and Occludin, two of the cell junction markers, they claimed to be increased by this protocol compared to iECs.

(6) Authors demonstrate that iRECs have a higher TEER than iECs. However, they do not provide the data on Bay-876 treatment. Bay-876 treatment significantly reduces the glucose uptake due to GLUT1 inhibition of iRECs, but does it have the same effect on iECs? It is crucial to assess the GLUT1 transport in both cell types if the glucose transport serves as a retinal endothelial phenotype.

(7) The authors claim in Figure S3A, that iRECs show increased Claudin-2 expression at cell-cell junctions compared to iECs. Later, in Figure S4C, they claim that the Claudin-2 expression of iRECs in diabetic conditions does not differ from the control. However, the shown representative images of Claudin-2 staining in iECs and diabetic iRECs look very similar. So either there is no Claudin-2 expression difference between iECs and iRECs, or the diabetic treatment of iRECs caused a reduced localization of Claudin-2 at cell-cell junctions.

(8) The authors do a transcriptomic analysis of iRECs, and iECs and compare them with iPSC-derived brain ECs. Of course, there are similarities (both ECs) and differences (different differentiation protocols). Would it not have been more meaningful to compare the iRECs and iECs with human retinal endothelial cells, since the focus of the paper is to prove that iRECs show more retinal identity than iECs?

(9) Revascularization experiments in the ischemic eye were conducted only with iRECs. To demonstrate the increased therapeutic potential of iRECs, it is important to compare them with iECs. Ideally, the therapeutic effect of iRECs should also be compared with that of primary mouse retinal endothelial cells.

(10) In Figure 5Di, the authors show the existence of a lumen in the vasculature. However it seems, that not every asterisk is indeed a lumen, some might be just holes in the vessels (especially the ones shown in the xy-image in which you can not prove that the lumen is enclosed by ECs).

(11) Are there any differences in marker expression between mesodermal PCs and iRPCs? The effect of increased Fz4 could be the same as in iRECs: the introduction of the ligand Norrin may upregulate Fz4 expression through a positive feedback loop, rather than proving the existence of a new cell subpopulation.

(12) The Permeability assay is missing in the methods.

(13) The used iPSC line contains EGFP-tagged ZO-1. However, the images are still referred to as immunofluorescence of ZO-1. Were these cells additionally stained with antibodies against ZO-1?

In summary, several critical issues need to be addressed before the paper can be considered for publication. The presented results might be interpreted as the Norrin impact on gene expression, TEER, and microvasculature formation, but they are not proof of successful iREC/PC differentiation protocol. Due to multiple methodological inconsistencies and a lack of results on multiple cell lines, I can not recommend the paper for publication.

Reviewer #3

(Report for the authors (Required))

This manuscript describes a method to derive human retinal endothelial cells (iRECs) from hiPSCs by activating the Wnt/ β -catenin signaling pathway, specifically through Norrin/Fz4 signaling. The authors claim that these iRECs recapitulate key molecular, phenotypic, and functional features of native retinal endothelial cells, including barrier function, responsiveness to diabetic retinopathy (DR)-like conditions, and in vivo vasculogenic potential in an oxygen-induced retinopathy (OIR) model. They further demonstrate the integration of iRECs with iPSC-derived retinal pericytes to form a perfusable, microphysiological iBRB-on-a-chip platform. This work is positioned within the fields of vascular biology, regenerative medicine, and ophthalmology, where robust, tissue-specific endothelial cell models are critical for disease modeling and therapeutic development.

The development of protocols aimed at generating endothelial cells with bona fide tissue-specific identities is an area of growing importance in vascular biology, with significant implications for both mechanistic studies and translational applications. In this context, the authors' efforts to define and validate a retinal-specific endothelial phenotype from hiPSCs address a timely and relevant question. The manuscript presents a technically ambitious and well-structured body of work, and the integration of iRECs into a microphysiological model is a notable strength.

However, based on the current data, the study does not yet establish that iRECs have acquired a definitive retina-specific identity that functionally distinguishes them from other iPSC-derived endothelial cells. Key aspects of tissue specificity—particularly at the transcriptomic, functional, and in vivo levels—would benefit from further support.

I have the following major concerns.

Major Concerns

1. The study does not provide an adequate dissection of the differentiation protocol components. The iREC protocol relies on sequential exposure to Norrin and vitronectin (Days 2–5), followed by RepSox (Days 5–8), but the rationale for this timing and factor selection is not well explained. It is unclear whether these cues act through their anticipated signaling pathways or how each contributes to the final phenotype. For example, it would be informative to compare iECs generated without Norrin and vitronectin but with RepSox, or vice versa. Without these controls, it is difficult to determine whether all three factors are necessary or if one alone is sufficient.
2. The point about Frizzled-4 expression and internalization is not sufficiently clear. The authors state that ~50% of pre-iRECs undergo Fz4 internalization (Fig. 1Ciii), and that ~66% of CD31+ pre-sorted iRECs express Fz4 (Fig. 1Civ), but it is unclear whether this reflects surface or total expression. Additionally, similar surface vs. total Fz4 analysis is not provided for control iECs. In Fig. 1Cii, the addition of Norrin appears to reduce the CD31+Fz4+ surface population—possibly due to internalization—but this interpretation remains speculative. A side-by-side comparison of surface and total Fz4 in both iRECs and iECs would help clarify whether Fz4 internalization is specifically induced by Norrin signaling or reflects general heterogeneity in the differentiation process.
3. Overall, there is limited evidence distinguishing iRECs from control iECs. The main phenotypic differences appear to be the expression of Claudin-2 and Occludin (Fig. 1), but these observations are not supported by quantitative measurements such as western blotting, flow cytometry, or mean fluorescence intensity from immunostaining. Without such data, it is difficult to assess the robustness and extent of the reported differences. On the transcriptomic side, the authors identify several differentially expressed genes—such as FZD4, IGF2, CLDN10, CLDN23, VTN, and FN1 (Fig. 3)—but do not benchmark these against primary HRECs or retina-specific vascular datasets. As a result, it remains unclear how closely iRECs approximate native retinal endothelial cells. It would also strengthen the conclusions to examine how these gene expression patterns change when Norrin/vitronectin or RepSox are individually excluded.
4. There is no evaluation of DR-like responses in control iECs. The authors show that iRECs respond to high glucose and hypoxia (Fig. 2D–E), recapitulating features of diabetic retinopathy. However, without performing the same experiments in iECs, it is unclear whether these responses are specific to the iREC phenotype or simply reflect a general property of iPSC-derived endothelial cells. This comparison is essential to support the claim that iRECs provide a tissue-relevant model for retinal vascular dysfunction.
5. The transcriptomic analysis lacks tissue-specific benchmarking. While the authors show that iRECs differ from iECs and exhibit Wnt pathway enrichment (Fig. 3), they do not compare these profiles to primary human RECs or to publicly available retinal vascular datasets. As a result, the claim that iRECs “recapitulate fundamental and developmental REC markers” feels overstated. The data suggest tissue specificity but do not confirm it. In addition, validating Wnt pathway activation at the protein level—such as through nuclear β -catenin localization—would further support the interpretation of pathway engagement.
6. The in vivo studies omit iECs as a therapeutic control. In the OIR model, iRECs reduce vaso-obliteration and neovascularization compared to PBS-treated eyes (Fig. 4), but without a direct comparison to iECs, it is not possible to determine whether the observed benefit is specific to the iREC phenotype. Including iECs as a control would have clarified whether iRECs offer superior or tissue-specific therapeutic potential. It would also have been informative to assess in vivo phenotypic maturation of the implanted cells using human-specific markers or transcriptomic analysis, to determine whether iRECs acquire retina-specific signatures after engraftment.

7. The microfluidic iBRB model lacks appropriate control comparisons. The authors present a perfusable iBRB-on-chip system using iRECs (Fig. 5), but without including iECs as a control, it is difficult to assess whether iRECs confer any specific functional or structural advantage. In addition, previous studies have shown that iPSC-derived ECs often require stromal or mural support to form stable vascular networks. It is unclear whether the iREC networks shown in Fig. 5A–D were formed in isolation or with supporting cells—this should be clarified.

8. The characterization of iPSC-derived retinal pericytes (iRPCs) is underdeveloped. Their retinal specificity is primarily inferred from Fz4/PDGFR β co-expression (Fig. 5F–G), but this conclusion appears premature. There is no comparison to control iPSC-derived pericytes or other mesenchymal populations, and no functional assays are provided to assess pericyte competence. Moreover, the protocol for iRPC generation is introduced with minimal validation, and the role of Norrin in their specification is not explored in depth. Also, the conclusion that the iREC–iRPC co-culture “recapitulates tissue-specific retinal-like vascular networks” is not adequately supported. While the structural organization is evident, the study does not show that this model performs better than alternative combinations, such as iECs with non-retinal pericytes. The authors could reasonably state that the system recapitulates physiological endothelial–pericyte interactions, but the tissue-specific aspect of the model is not convincingly demonstrated.

Minor Concerns

In Fig. 1, it would be helpful to report the efficiency of the Fz4+ sorting step. Providing post-sort purity (e.g., via flow cytometry) would help assess the robustness of the protocol and the homogeneity of the iREC population.

Decision Letter:

Dear Prof Gerecht,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Derivation of functional retinal endothelial cells from human pluripotent stem cells for therapeutics and modeling". The manuscript has been seen by 3 experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate the importance of the work. However, they raise serious concerns about the overall significance of the study in the absence of clear evidence supporting the nature of the developed iRECs and their actual superiority in mimicking the relevant tissue respect to more easily accessible alternatives, and provide useful suggestions for improvement.

We hope that with substantial further effort you can address the criticisms, increase the strength of the evidence, and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

- * Prove, via direct comparison, the fundamental advancements provided by the iRECs over iECs.
- * Demonstration that the iRECs can more precisely model the retina EC behaviour.
- * Provide a rational, supported by relevant controls for justifying the chosen growth factors for the protocol.
- * Add quantitative assessments and the many essential controls as per the quasi-unanimous concerns of the reviewers.

When you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from all reviewers, the [reporting summary](https://www.nature.com/authors/policies/ReportingSummary.pdf), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

- *Please submit your revised manuscript in a word doc or LaTeX format.
- * Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).
- * If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).
- * If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).
- * Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.
- * The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will

the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We expect that you will be able to resubmit the manuscript within 25 weeks of receiving this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

[Redacted]

Reviewer #1 (Report for the authors (Required)):

Summary

In this manuscript, Lin et al develop a protocol to differentiate retina-like ECs from hiPSCs and characterize them transcriptionally, and functionally in vitro and in vivo. They achieve this by addition of Norrin and vitronectin to the differentiation and 2 rounds of sorting for CD31 and Fz4 to induce iREC 'specification'. "generic" EC –not treated with Norrin and vitronectin–were used as control for retina-specific assessments. Authors used 2 different hiPSC lines and compared iRECs to primary retinal ECs. They tested the effects of HG media and hypoxia on iREC junctions and TEER and used a mouse model of oxygen-induced retinopathy to investigate iREC incorporation into the developing eye vasculature where PBS injection to the contralateral eye was used as control. Finally, they used microphysiological systems to seed iREC and pericytes to investigate inner blood-retinal barrier properties in vitro.

Degree of advance

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Major criticism

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(3) Figure 1D demonstrates the differences in morphology and marker expression of iECs and iREC. The morphology of iECs appears healthier: cells are uniformly shaped and properly aligned with each other. The round and irregular shape of iRECs, on the other hand, reminds features of senescence or dysfunctional ECs.

(4) Figure S2 demonstrates the staining and morphology of HRECs. The cell density appears low and cell morphology is generally unhealthy. Authors should consider excluding these cells from the analysis since results received with unhealthy cells are unreliable.

(5) Figure S2C should prove the robustness of the protocol by using another hiPSC line to differentiate iRECs. However, the authors do not show the expression of ZO-1 and Occludin, two of the cell junction markers, they claimed to be increased by this protocol compared to iECs.

(6) Authors demonstrate that iRECs have a higher TEER than iECs. However, they do not provide the data on Bay-876 treatment. Bay-876 treatment significantly reduces the glucose uptake due to GLUT1 inhibition of iRECs, but does it have the same effect on iECs? It is crucial to assess the GLUT1 transport in both cell types if the glucose transport serves as a retinal endothelial phenotype.

(7) The authors claim in Figure S3A, that iRECs show increased Claudin-2 expression at cell-cell junctions compared to iECs. Later, in Figure S4C, they claim that the Claudin-2 expression of iRECs in diabetic conditions does not differ from the control. However, the shown representative images of Claudin-2 staining in iECs and diabetic iRECs look very similar. So either there is no Claudin-2 expression difference between iECs and iRECs, or the diabetic treatment of iRECs caused a reduced localization of Claudin-2 at cell-cell junctions.

(8) The authors do a transcriptomic analysis of iRECs, and iECs and compare them with iPSC-derived brain ECs. Of course, there are similarities (both ECs) and differences (different differentiation protocols). Would it not have been more meaningful to compare the iRECs and iECs with human retinal endothelial cells, since the focus of the paper is to prove that iRECs show more retinal identity than iECs?

(9) Revascularization experiments in the ischemic eye were conducted only with iRECs. To demonstrate the increased therapeutic potential of iRECs, it is important to compare them with iECs. Ideally, the therapeutic effect of iRECs should also be compared with that of primary mouse retinal endothelial cells.

(10) In Figure 5Di, the authors show the existence of a lumen in the vasculature. However it seems, that not every asterisk is indeed a lumen, some might be just holes in the vessels (especially the ones shown in the xy-image in which you can not prove that the lumen is enclosed by ECs).

(11) Are there any differences in marker expression between mesodermal PCs and iRPCs? The effect of increased Fz4 could be the same as in iRECs: the introduction of the ligand Norrin may upregulate Fz4 expression through a positive feedback loop, rather than proving the existence of a new cell subpopulation.

(12) The Permeability assay is missing in the methods.

(13) The used iPSC line contains EGFP-tagged ZO-1. However, the images are still referred to as immunofluorescence of ZO-1. Were these cells additionally stained with antibodies against ZO-1?

In summary, several critical issues need to be addressed before the paper can be considered for publication. The presented results might be interpreted as the Norrin impact on gene expression, TEER, and microvasculature formation, but they are not proof of successful iREC/PC differentiation protocol. Due to multiple methodological inconsistencies and a lack of results on multiple cell lines, I can not recommend the paper for publication.

Reviewer #3 (Report for the authors (Required)):

This manuscript describes a method to derive human retinal endothelial cells (iRECs) from hiPSCs by activating the Wnt/ β -catenin signaling pathway, specifically through Norrin/Fz4 signaling. The authors claim that these iRECs recapitulate key molecular, phenotypic, and functional features of native retinal endothelial cells, including barrier function, responsiveness to diabetic retinopathy (DR)-like conditions, and in vivo vasculogenic potential in an oxygen-induced retinopathy (OIR) model. They further demonstrate the integration of iRECs with iPSC-derived retinal pericytes to form a perfusable, microphysiological iBRB-on-a-chip platform. This work is positioned within the fields of vascular biology, regenerative

medicine, and ophthalmology, where robust, tissue-specific endothelial cell models are critical for disease modeling and therapeutic development.

The development of protocols aimed at generating endothelial cells with bona fide tissue-specific identities is an area of growing importance in vascular biology, with significant implications for both mechanistic studies and translational applications. In this context, the authors' efforts to define and validate a retinal-specific endothelial phenotype from hiPSCs address a timely and relevant question. The manuscript presents a technically ambitious and well-structured body of work, and the integration of iRECs into a microphysiological model is a notable strength.

However, based on the current data, the study does not yet establish that iRECs have acquired a definitive retina-specific identity that functionally distinguishes them from other iPSC-derived endothelial cells. Key aspects of tissue specificity—particularly at the transcriptomic, functional, and in vivo levels—would benefit from further support.

I have the following major concerns.

Major Concerns

1. The study does not provide an adequate dissection of the differentiation protocol components. The iREC protocol relies on sequential exposure to Norrin and vitronectin (Days 2–5), followed by RepSox (Days 5–8), but the rationale for this timing and factor selection is not well explained. It is unclear whether these cues act through their anticipated signaling pathways or how each contributes to the final phenotype. For example, it would be informative to compare iECs generated without Norrin and vitronectin but with RepSox, or vice versa. Without these controls, it is difficult to determine whether all three factors are necessary or if one alone is sufficient.
2. The point about Frizzled-4 expression and internalization is not sufficiently clear. The authors state that ~50% of pre-iRECs undergo Fz4 internalization (Fig. 1Ciii), and that ~66% of CD31+ pre-sorted iRECs express Fz4 (Fig. 1Civ), but it is unclear whether this reflects surface or total expression. Additionally, similar surface vs. total Fz4 analysis is not provided for control iECs. In Fig. 1Cii, the addition of Norrin appears to reduce the CD31+Fz4+ surface population—possibly due to internalization—but this interpretation remains speculative. A side-by-side comparison of surface and total Fz4 in both iRECs and iECs would help clarify whether Fz4 internalization is specifically induced by Norrin signaling or reflects general heterogeneity in the differentiation process.
3. Overall, there is limited evidence distinguishing iRECs from control iECs. The main phenotypic differences appear to be the expression of Claudin-2 and Occludin (Fig. 1), but these observations are not supported by quantitative measurements such as western blotting, flow cytometry, or mean fluorescence intensity from immunostaining. Without such data, it is difficult to assess the robustness and extent of the reported differences. On the transcriptomic side, the authors identify several differentially expressed genes—such as FZD4, IGF2, CLDN10, CLDN23, VTN, and FN1 (Fig. 3)—but do not benchmark these against primary HRECs or retina-specific vascular datasets. As a result, it remains unclear how closely iRECs approximate native retinal endothelial cells. It would also strengthen the conclusions to examine how these gene expression patterns change when Norrin/vitronectin or RepSox are individually excluded.
4. There is no evaluation of DR-like responses in control iECs. The authors show that iRECs respond to high glucose and hypoxia (Fig. 2D–E), recapitulating features of diabetic retinopathy. However, without performing the same experiments in iECs, it is unclear whether these responses are specific to the iREC phenotype or simply reflect a general property of iPSC-derived endothelial cells. This comparison is essential to support the claim that iRECs provide a tissue-relevant model for retinal vascular dysfunction.
5. The transcriptomic analysis lacks tissue-specific benchmarking. While the authors show that iRECs differ from iECs and exhibit Wnt pathway enrichment (Fig. 3), they do not compare these profiles to primary human RECs or to publicly available retinal vascular datasets. As a result, the claim that iRECs “recapitulate fundamental and developmental REC markers” feels overstated. The data suggest tissue specificity but do not confirm it. In addition, validating Wnt pathway activation at the protein level—such as through nuclear β -catenin localization—would further support the interpretation of pathway engagement.
6. The in vivo studies omit iECs as a therapeutic control. In the OIR model, iRECs reduce vaso-obliteration and neovascularization compared to PBS-treated eyes (Fig. 4), but without a direct comparison to iECs, it is not possible to determine whether the observed benefit is specific to the iREC phenotype. Including iECs as a control would have clarified whether iRECs offer superior or tissue-specific therapeutic potential. It would also have been informative to assess in vivo phenotypic maturation of the implanted cells using human-specific markers or transcriptomic analysis, to determine whether iRECs acquire retina-specific signatures after engraftment.
7. The microfluidic iBRB model lacks appropriate control comparisons. The authors present a perfusable iBRB-on-chip system using iRECs (Fig. 5), but without including iECs as a control, it is difficult to assess whether iRECs confer any specific functional or structural advantage. In addition, previous studies have shown that iPSC-derived ECs often require stromal or mural support to form stable vascular networks. It is unclear whether the iREC networks shown in Fig. 5A–D were formed in isolation or with supporting cells—this should be clarified.
8. The characterization of iPSC-derived retinal pericytes (iRPCs) is underdeveloped. Their retinal specificity is primarily inferred from Fz4/PDGF β co-expression (Fig. 5F–G), but this conclusion appears premature. There is no comparison to

control iPSC-derived pericytes or other mesenchymal populations, and no functional assays are provided to assess pericyte competence. Moreover, the protocol for iRPC generation is introduced with minimal validation, and the role of Norrin in their specification is not explored in depth. Also, the conclusion that the iREC–iRPC co-culture “recapitulates tissue-specific retinal-like vascular networks” is not adequately supported. While the structural organization is evident, the study does not show that this model performs better than alternative combinations, such as iECs with non-retinal pericytes. The authors could reasonably state that the system recapitulates physiological endothelial–pericyte interactions, but the tissue-specific aspect of the model is not convincingly demonstrated.

Minor Concerns

In Fig. 1, it would be helpful to report the efficiency of the Fz4+ sorting step. Providing post-sort purity (e.g., via flow cytometry) would help assess the robustness of the protocol and the homogeneity of the iREC population.

Version 1:

Reviewer comments:

Reviewer #1

(Report for the authors (Required))

The authors have conducted key additional experiments and provided new data supporting the superior performance of iRECs (compared to generic iECs) in in vitro disease modeling. Several concerns raised in the previous review have been addressed. However, although the in vitro benefits of iRECs are now well demonstrated, their claimed superiority in vivo remains unsubstantiated. A number of important issues persist:

1. Vessel permeability assessment in the MPS

The method used to assess vessel permeability is unclear and requires further clarification. The authors state that they filled the hydrogel with fluorescent tracer and quantified fluorescence intensity changes within the vascular network, which, if interpreted correctly, reflects reverse permeability. If this is indeed the intention, representative images showing differences within the vasculature should be provided, as the current images illustrate only the interstitium. Even if images reveal differences, the relevance of this assay to physiological permeability remains questionable and should be justified.

2. Discrepancies between Fig. S15 Ei/Eiii and Fig. 5Ei/Ji

Figures S15 Ei and Eiii look markedly different from Fig. 5Ei and Ji. The authors should explain this discrepancy. Additionally, why was the perfusion method, which is standard in the field not used to assess permeability directly?

3. Gene expression comparisons between HRECs, iECs, and iRECs

The gene expression analysis shows that iECs and iRECs are far more similar to each other than either is to HRECs. This is concerning, as many genes exhibit an inverse expression pattern relative to HRECs. The authors should address this more thoroughly, discussing implications for the retinal identity and fidelity of iRECs.

4. Claims regarding “enhanced retina-specific reparative potential of iRECs”

The data do not sufficiently support this conclusion.

- In Fig. S14, the iEC networks (panel Fi) appear far more robust than either PBS or iREC conditions (panel E). Based on visual assessment and quantitative parameters, there are very few differences between iECs and iRECs in reparative potential.

- The two experimental batches differ substantially.

- o Batch 1 (original submission): PBS vs iREC, with VO = 20% in PBS controls.

- o Batch 2 (revision): PBS vs iEC, with VO = 10% in PBS controls.

This suggests a substantially milder insult in Batch 2, which may mask differences between PBS and iECs. Importantly, variability in PBS controls is 2× between batches, whereas variability in treated groups (iEC/iREC) is lower, indicating high model variability overall.

- Only two parameters showed significant differences for iRECs: network segment volume (noting that iECs were nearly significant, $p = 0.059$ —was power calculated?) and vessel diameter, whose relevance to regeneration is unclear.

- VO, NV, and Dextran fluorescence intensity differed significantly for both iECs and iRECs, while branch length and surface area did not differ for either group. This pattern suggests more similarities than differences between iECs and iRECs in vivo.

Taken together, these findings may reflect assay variability rather than a true enhanced reparative potential of iRECs in comparison to iECs. While the authors were overall cautious in their claims, stating mostly that iRECs have a regenerative ability, the authors are encouraged to either provide additional evidence through a direct head-to-head comparison of iECs and iRECs within the same experimental batch, or remove claims of in vivo superiority (page 34, last sentence) and include this as a limitation of the study.

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The perfusion data are not convincing. There appears to be as much (or more) red signal outside the vessels as inside. The small region where red overlaps with green seems localized and potentially artifactual. If the vessels are leaky—making perfusion difficult to assess—this should be explicitly addressed. The authors should clarify:

- Why such extensive extravascular red signal is present,
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General Comments

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- All statistical methods and sample sizes (n) should be included in each figure legend to allow proper evaluation of the results.

Reviewer #2

(Report for the authors (Required))

The authors have followed our suggestions.

Reviewer #3

(Report for the authors (Required))

The authors have responded thoroughly to the prior critiques, and the manuscript is clearly improved. The added controls, functional comparisons, and clarification of claims strengthen the overall rigor. The conclusions are now more measured, and the key outcomes are supported by the data. I am satisfied with the revisions. One small point that could be acknowledged in the Discussion is that, at a global transcriptomic level, iRECs remain closer to iECs than to primary HRECs, which is expected at this stage. This does not detract from the significance of the work but would help to frame the findings realistically.

Decision Letter:

Dear Sharon,

Thank you again for submitting to *Nature Biomedical Engineering* your revised manuscript, "Derivation of functional retinal endothelial cells from human pluripotent stem cells for therapeutics and modeling". The manuscript has been seen by the original 3 experts whose reports you will find at the end of this message. You will see that the reviewers appreciate the additional work, and that they raise a few more technical criticisms that we hope you will be able to address.

As before, when you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from all reviewers, the [reporting summary](https://www.nature.com/authors/policies/ReportingSummary.pdf), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

- * Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).
- * If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).
- * If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).
- * Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.
- * The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).
- * Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We expect that you will be able to resubmit the manuscript within 15 weeks of receiving this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical*

We hope that you will find the referee reports helpful when revising the work, which we look forward to receive. Please do not hesitate to contact me should you have any questions.

Best wishes,

[Redacted]

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Version 2:

Reviewer comments:

Reviewer #1

(Report for the authors (Required))

The authors have appropriately addressed the concerns. I congratulate the authors on the beautiful work.

Decision Letter:

Dear Sharon,

Thank you for submitting your revised manuscript "Derivation of functional retinal endothelial cells from human pluripotent stem cells for therapeutics and modeling" (NBME-25-0785B). Having consulted with Reviewers #1, (whose comment you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in Nature Biomedical Engineering, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements. Please do not upload the final materials and make any revisions until you receive this additional information from us.

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Sincerely,

[Redacted]

Reviewer #1 (Report for the authors (Required)):

The authors have appropriately addressed the concerns. I congratulate the authors on the beautiful work.

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Reviewer comments:

Reviewer #1:

Degree of advance

The field of endothelial organotypic identity and plasticity has recently attracted considerable attention. Seminal questions related to EC organotypic identity in engineered tissues and regenerative medicine are unanswered. Key questions relate to whether ECs can adopt the organ-specific characteristics of their implantation site or if organ-specific ECs are required for tissue/organ function in transplantation. Papers addressing these seminal questions should draw significant attention and be highly cited. While Lin et al tangentially address some aspects of this exciting area of research, the lack of appropriate controls (generic ECs) for functional readouts in both in vivo and in vitro models have dampened the enthusiasm for the findings.

There is certainly a methodological advance: the development of an EC differentiation protocol to generate retina-like ECs. However, not enough evidence was given, in its present form, to provide significant fundamental, mechanistic, or performance-wise advances – which would increase significantly the impact of this paper.

Implication of the findings

As stated by the authors, 'there is an increasing need to develop tissue-specific ECs for more precise disease modeling and effective tissue regeneration and repair'. However, there was no demonstration in this manuscript that the retina-like ECs more precisely model the retina EC behavior or show tissue-specific function in vitro and in vivo when compared to generic ECs.

While generating retina-like ECs and studying their general characteristics certainly has merit, the greatest advancement would be to provide evidence that it is advantageous to use tissue-specific ECs (in this case iRECs) in vitro (as even native primary tissue-specific cells lose phenotype in culture) and in vivo compared to 'generic' ECs or cells from other tissues. Given that the prevailing dogma is that ECs are plastic and can adapt to the local environment, the significant higher cost and time needed to differentiate tissue-specific ECs needs to be justified. However, authors failed to provide convincing evidence of the advantages of using iREC, as most assays performed lacked a direct comparison to generic ECs or ECs of different origins. Without this, the advancement to the field is modest and future adoption by others will be limited. Towards that goal, authors should provide appropriate controls for functional assays.

Major criticism

1- Lack of 'generic' EC controls in both in vitro and cell transplantation assays limits the conclusions

We thank the reviewer for highlighting this important point. To strengthen our study, we included iECs as controls in several key assays to further demonstrate the biological fidelity of the iRECs and their ability to model DR and repair the ischemic retina. iECs were incorporated as a control in:

- GLUT1 and P-gp transporter assays (New Fig. S6C-D),
- Permeability and TEER assay (New Fig. S6B),
- All DR modeling experiments (New Fig. S8E-G, and S9B),
- The OIR mouse model (New Fig. S14), and
- The MPS study (New Fig. S16C-E).

These experiments have enabled us to compare iRECs to iECs to better highlight the unique retinal-specific properties of iRECs and their overall robustness.

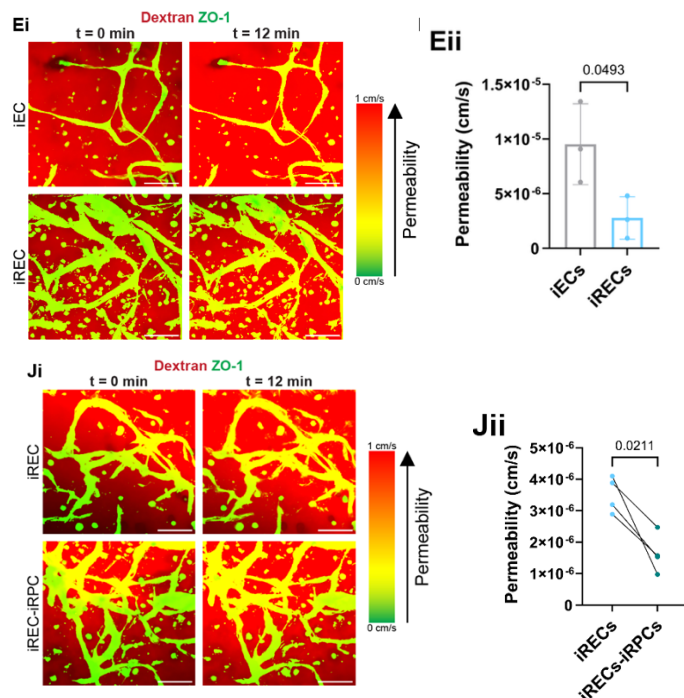
These comparisons consistently demonstrate that iRECs possess retinal-specific properties and functionalities. *In vitro*, they exhibit robust GLUT1 and p-gp transporter activity, enhanced barrier function with higher TEER and lower permeability, and greater sensitivity to DR-associated stress with pronounced reductions in barrier properties and junctional protein expression. *In vivo*, iRECs more effectively restore vascular network morphology in the OIR model. In the MPS, iREC networks possess increased junctional protein expression and display lower permeability than iEC networks, reflecting physiologically accurate barrier function.

Collectively, these results confirm that iRECs outperform generic iECs in modeling retinal-specific vascular phenotypes and DR, reinforcing the biological fidelity and utility of the iREC platform.

2- It is unclear why the authors elected to test the vessel formation in MPS and not provide assessment of barrier function under fluid flow – which, as they point out themselves “enabling the study of tissue function” and this is the best application of MPS that is also relevant for their hypothesis. iRECs permeability coefficient should be provided and compared to iECs in the chip, under flow, to assess if they indeed enable improved barrier function. While TEER is a good method, it lacks functional assessment, which can be achieved in the chip (see Pham Lab on a chip 2017, Chen lam Chip 2023 etc.).

We thank the reviewer for this important comment. We included a permeability assay to assess the barrier function and establish the permeability coefficient of iECs, iRECs, and iREC-iRPCs within the MPS. To quantify permeability, our method uses a slightly modified approach based on an established study from Hajal et al., Nat. Protoc., 2022. Briefly, 20 μ L of transport buffer was delivered to one media channel and 70 μ L of transport buffer containing 100 μ g/mL 70 kDa rhodamine dextran to the opposite channel. This generated interstitial flow, enabling rhodamine dextran to perfuse and fill the collagen type-I hydrogel. Once the hydrogel was homogeneously filled, imaging began (t_0), and the same location was imaged 12 minutes later to complete the assay. Fluorescence intensity changes within the vascular networks were quantified over time to determine permeability, rather than measuring the surrounding hydrogel.

Using this assay, iREC networks were successfully perfused by 70 kDa rhodamine dextran and exhibited significantly lower permeability compared to iEC networks (New Fig. 5E). Co-culture with iRPCs further enhanced barrier properties, reducing 70 kDa rhodamine dextran permeability to levels consistent with *in vivo* measurements (New Fig. 5J). Collectively, these findings demonstrate that incorporation of iRPCs into the iBRB MPS strengthens EC barrier function and support the formation of physiological relevant vascular networks.



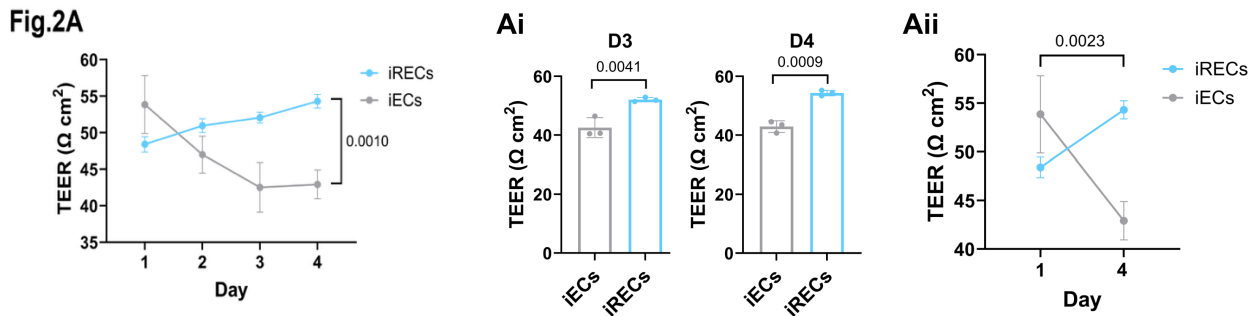
3- On TEER: what was the media used for the experiments? Methods describe that iECs were cultured in media with 50ng/ml of VEGF while iRECs in the same media but without VEGF – see excerpt below. Given that VEGF is well known for inducing EC permeability, differences in TEER could be due to the different culture media used. Results must be provided for assays performed with the same media for all cells.

“The purified CD31+ iECs were plated on a type-I collagen-coated plate and expanded and maintained in ECGM supplemented with 10 μ M SB-431542, 50 ng/ml VEGF-A, and 10 μ M Y-27632. After 24 hours, the media was changed to remove Y-27632. After that, media was changed every other day.”

“iRECs were plated on a type-I collagen-coated plate and expanded and maintained in ECGM supplemented with 10 μ M RepSox, 10% platelet poor human serum (Millipore Sigma), and 10 μ M Y-27632. After 24 hours, the media was changed to remove Y-27632 and then the media was changed every other day.”

We appreciate the reviewer’s comment. We reperformed the TEER and permeability assay with both iRECs and iECs cultured in iREC media. The methods section has been updated to include these new details.

With the new conditions, we observed higher TEER in the iRECs relative to the iECs across the four days (**Fig. 2A**), displaying enhanced barrier properties. We specifically identified that the iRECs possessed increased TEER values on day 3 and day 4 (**New Fig. S6Ai-ii**), suggesting a physiologically relevant maturation of iREC barrier integrity over time absent from iECs. This validates that the iRECs contain high barrier functionality consistent with the iBRB, supporting their biological fidelity and utility.

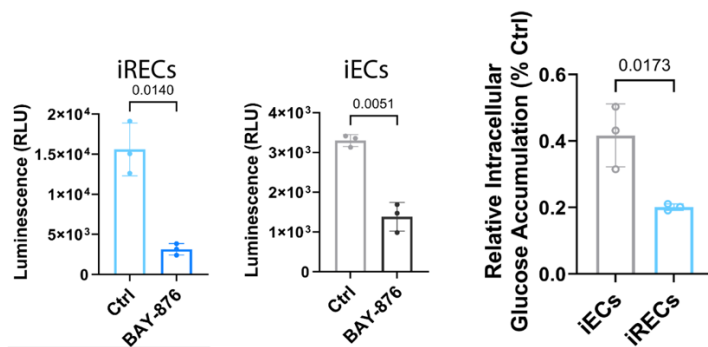


4- There are not enough details in the methods to reproduce the findings, so methods need to be improved substantially. Cell seeding density at thawing and passaging and for assays need to be provided, how many cells were collected in 100ul of FACs buffer, etc.

We thank the reviewer for this comment. We have updated the methods section to include more detailed information, including cell numbers used during stem cell culture, differentiation and the various assays.

5- It would be good to have a comparison between iREC and iECs for glut1 inhibition to show specificity

We thank the reviewer for this comment. We added new Fig. S6C to include iEC in the GLUT1 transporter assay. The GLUT1 receptor plays a crucial role in the retina. The GLUT1 transporter is the main facilitator of glucose transport, which is critical to meeting the energy demand in the retina. When treated with glucose, iRECs exhibit a 5-fold higher baseline glucose uptake level (1.5×10^4 vs. 3×10^3) than iECs (Fig. 2B and New Fig. S6Ci). When the iRECs were treated with the highly selective GLUT1 inhibitor, Bay-876, glucose uptake in iRECs was reduced by 80% while iEC uptake was lowered by only 60% (Fig. S6Cii). The greater GLUT1 activity and transporter functionality within iRECs supports their retinal specificity.



6- Fig 2 Cii, Eiii; S3a, b, Fig 5C are not really useful as it is difficult to draw conclusions from 1 image. For rigour, assessments must be provided as in Fig 2Dii.

We thank the reviewer for this comment. We agree that quantification is essential for rigor. We have now added quantification for Fig. 2Cii, 2Eiii, S3A, S3B (New Fig. S7A-B), and 5C (New Fig. S15D).

These analyses reveal that iREC networks exhibit elevated membrane-associated ZO-1, Claudin-2, Claudin-5, and β -catenin at cell-cell contacts relative to iEC networks, consistent with their iBRB-specific phenotype and enhanced barrier properties (TEER, permeability, and stability; New Fig. S7A). In contrast, canonical EC markers—CD31, VE-Cadherin, UEA1—show comparable expression between iRECs and iECs, confirming endothelial identity (New Fig. S7B).

Under diabetic retinopathy (DR) conditions, 3D iREC vascular networks showed decreased junctional expression of ZO-1, CD31, VE-Cadherin, Claudin-5, and β -catenin, indicating compromised barrier function, whereas Claudin-2 remained unchanged, consistent with prior in vivo observations (New Fig. S9A).

Furthermore, in MPS, iRECs consistently displayed higher junctional ZO-1, Claudin-2, and β -catenin than iECs (New Fig. S15D), reinforcing their retinal-specific barrier integrity and phenotypic accuracy.

Fig. S7. 3D iEC and iREC vascular networks

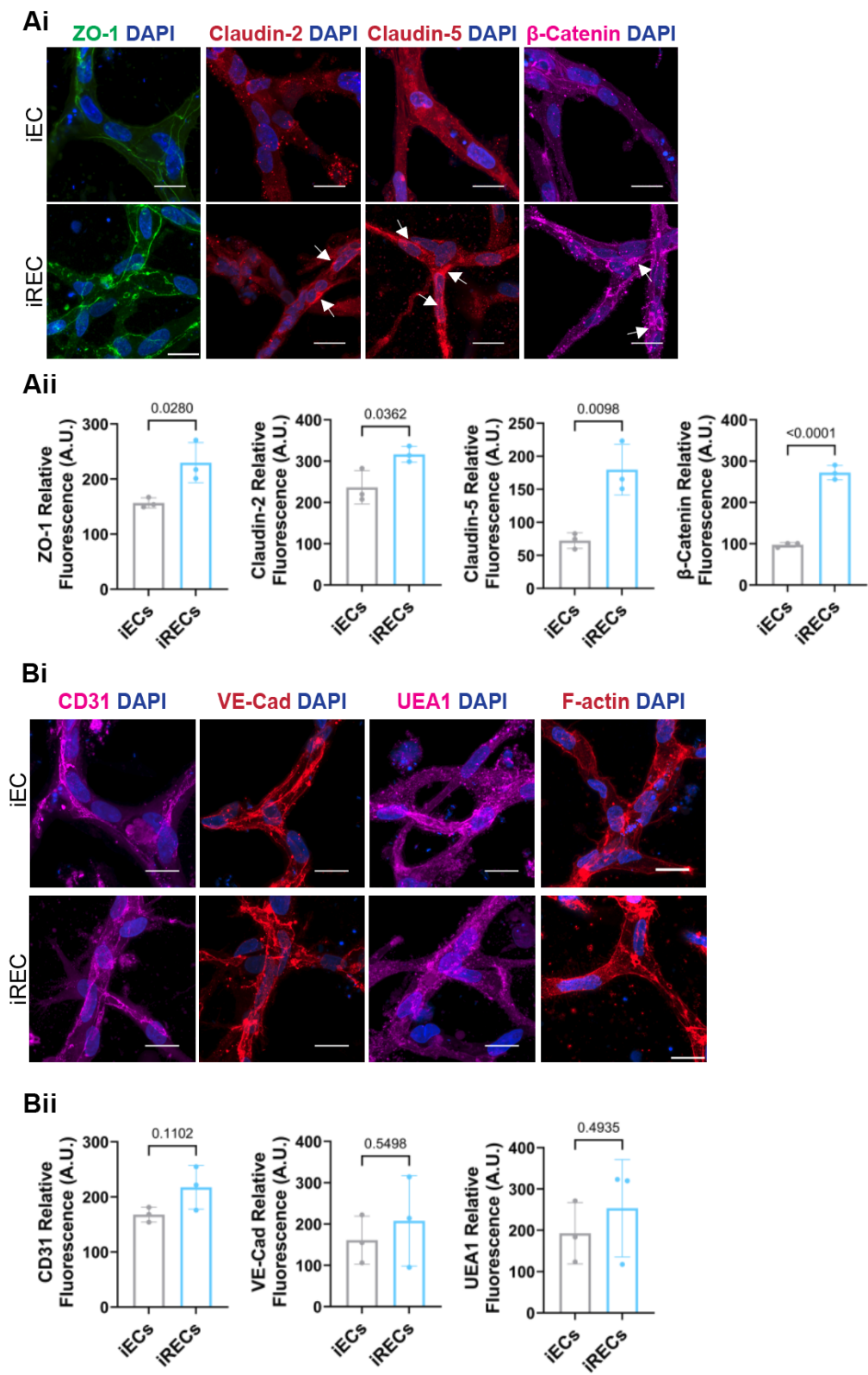


Fig. S9. 3D iREC vascular networks with or without diabetic treatment

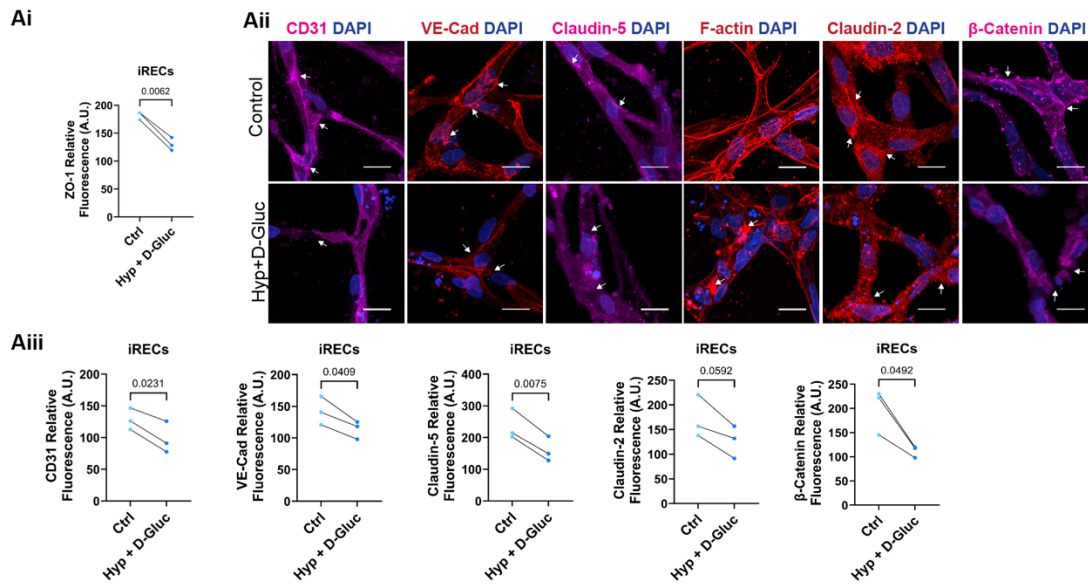
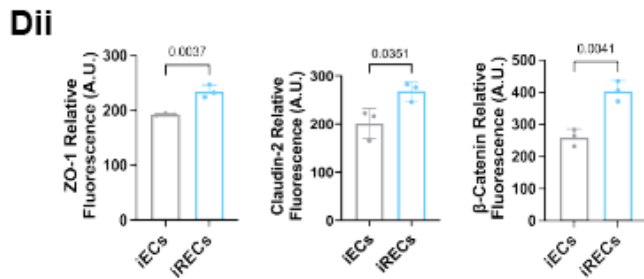


Fig. S15. Quantification of junctional protein expression in iEC and iREC vascular networks within MPS



7- Diabetic retinopathy conditions used in the manuscript are extreme: 30mM of glucose is extremely high and then there's the presence of 1% O₂ – for up to 72h. I am amazed any cells were still alive. Assessment of cell viability (e.g. live dead stain) would be useful to understand the extent of the DR treatment. Authors should also provide a better justification for using such an extreme model. Moreover, comparison with iECs should be done. Are iRECs better at modeling diabetic retinopathy? In which aspects? This would be extremely valuable for the field and highlight the advantages of using tissue-specific ECs.

We thank the reviewer for this thoughtful comment. The glucose concentration used to recapitulate DR was based on method previously utilized within the literature for *in vitro* DR modeling (Maurissen et al., Nat Commun, 2024; Eyre et al., Exp Eye Res, 2020; Kaur et al., Am J Physiol Cell Physiol, 2023; Chandrakumar et al., Diabetes, 2024). A glucose concentration of 30 mM resembles grade 4 serum hyperglycemia, a life-threatening condition that necessitates immediate treatment. Modeling this stage of hyperglycemia with our iRECs and systems can help accelerate therapeutic development to improve treatment efficacy.

To address the concern that this extreme condition could lead to decrease cell viability, we assessed cell viability in 2D using flow cytometry. We observed comparable survival between iRECs and iECs under DR conditions (New Fig. S8A), confirming that the treatment is not cytotoxic. Functional and structural analysis in both 2D and 3D cultures revealed that iRECs are more sensitive to DR-associated stress than iECs (New Fig. S9). iRECs showed significantly reduced TEER, decreased junctional protein expression (ZO-1, CD31, VE-Cadherin, Claudin-5, β -catenin), and decreased network morphology (Fig. 2D-E, New Fig. S9A, and New Fig. S8D). In contrast, iECs did not contain significant differences in TEER at individual time points and had reduced changes in TEER compared to the iRECs (New Figs. S8E-F). Initial testing with iECs in 2D demonstrate that there is not a difference in ZO-1, Claudin-5, and VE-Cadherin junctional protein expression between DR and control conditions (N = 1, New Fig. S8G, not in the manuscript) Corroborating this, iEC networks treated with DR conditions possessed branch lengths, surface areas, network volumes, and lumen

diameters consistent with the control groups (New Fig. S9B). Similarly, while CD31, VE-Cadherin, Claudin-5, and β -catenin protein expression are trending toward a decrease in these DR iEC networks, the reduction is not statistically significant (New Fig. S9C).

These results indicate that iRECs faithfully recapitulate structural and functional features of DR, whereas generic iECs show limited responsiveness, highlighting the retinal specificity and modeling advantage of iRECs.

Fig. S8A. Cell viability analysis of control and diabetic-treated iECs and iRECs.

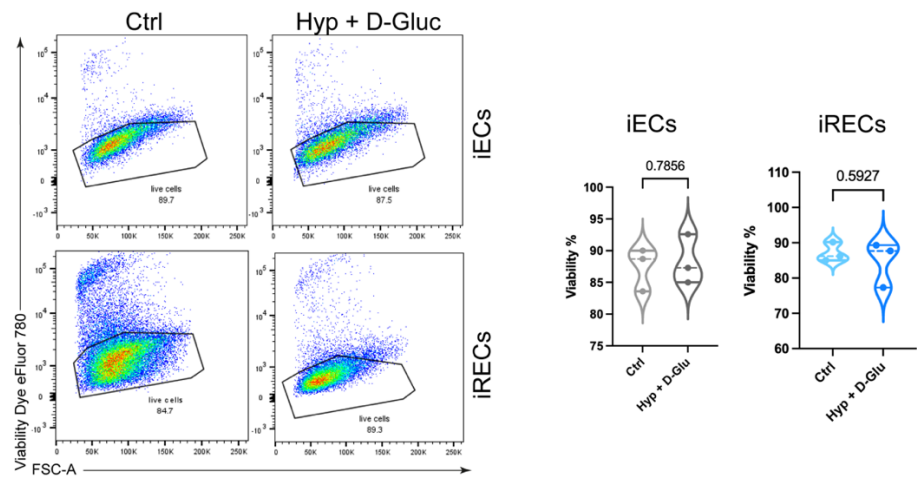


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment

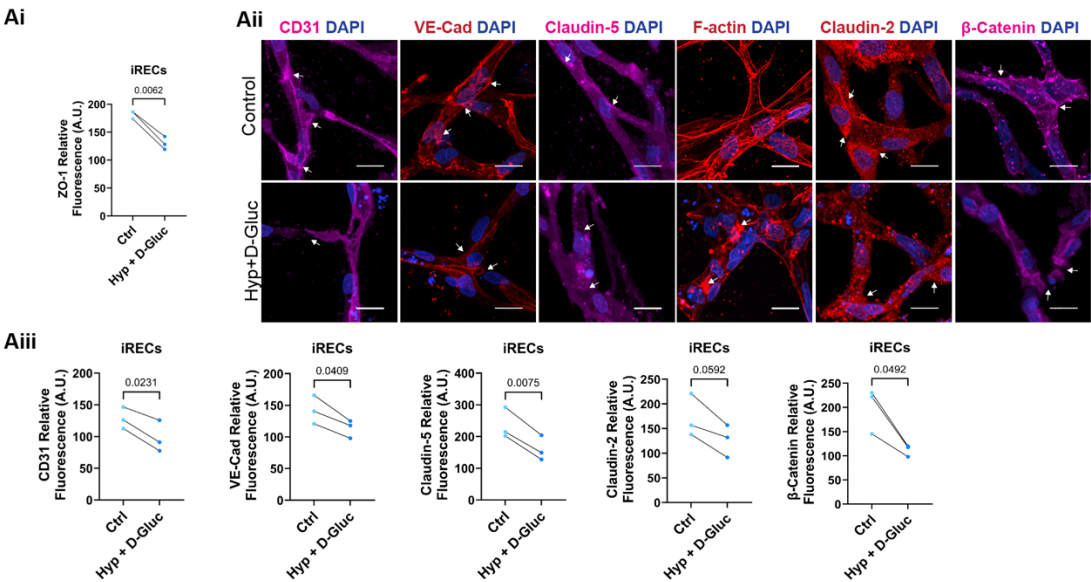


Fig. 2Diii and Fig. S8D. TEER measurement of iRECs with or without DR treatment

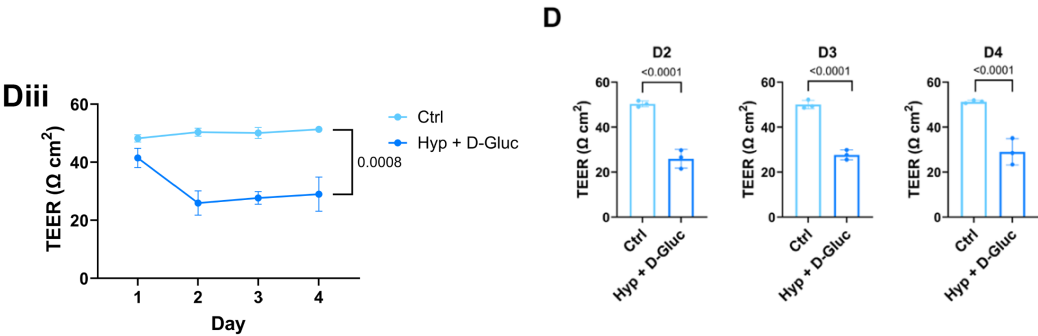


Fig. S8E-F. TEER measurement of iECs with or without DR treatment. And relative TEER change in iECs and iRECs after DR treatment.

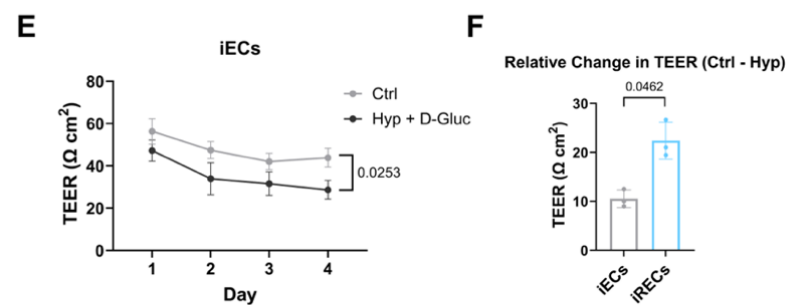


Fig.S8G. 2D iECs with or without DR treatment (Data not shown in the manuscript)

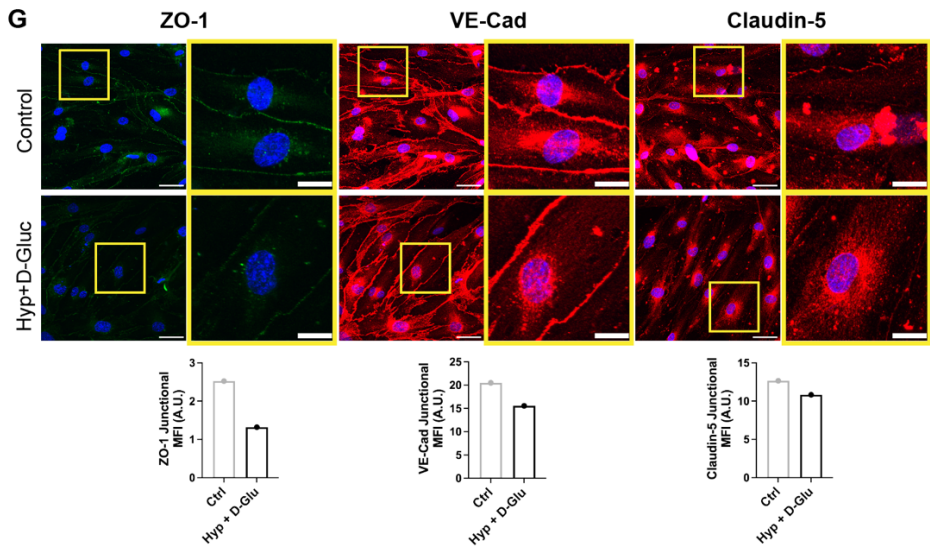


Fig.S9B. Morphology analysis or iEC vascular networks with or without DR treatment

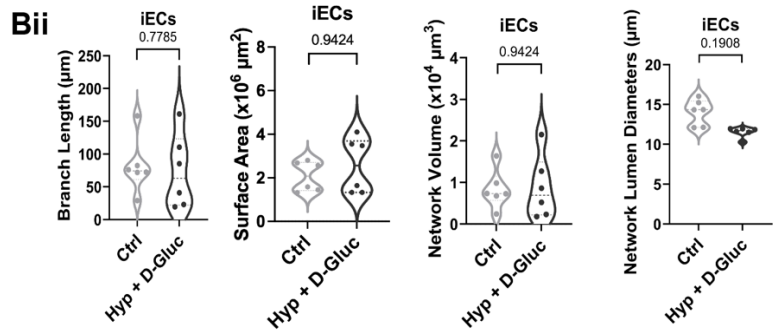
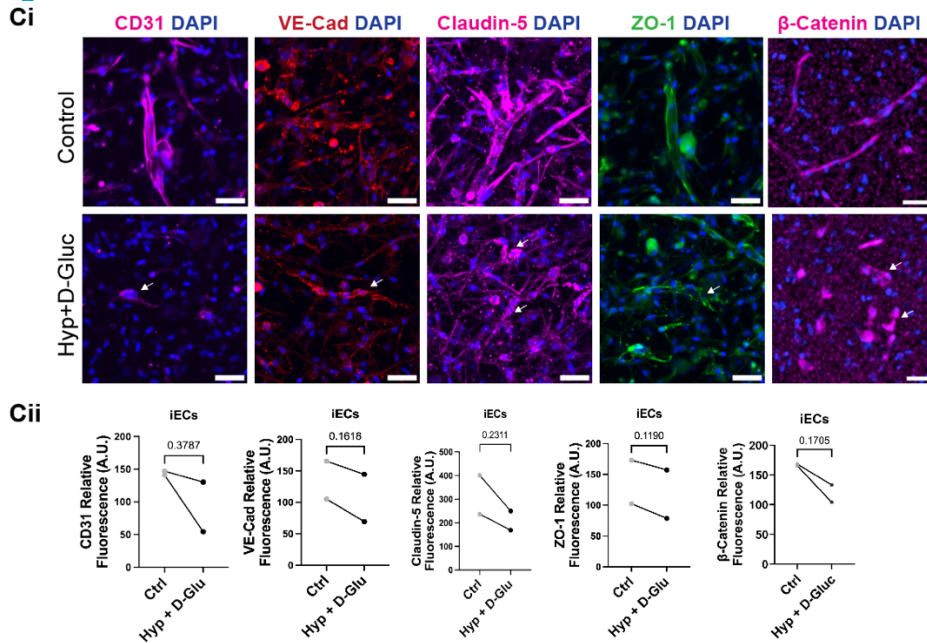


Fig. S9C. 3D iEC vascular networks with or without diabetic treatment



8- Was the hypothesis that DR would induce loss of Claudin-2 junctions? Is that why only iRECs were used in this assay? Is this what has been reported in DR in vivo and that the authors have tried to recapitulate?

We thank the reviewer for this question and helpful comment. DR is characterized by a breakdown of the iBRB, resulting in decreased EC barrier function and disrupted cell-cell junctions. Interestingly, an elegant *in vivo* study found that Claudin-2 protein expression does not change in physiological and DR conditions (Luo et al., Invest Ophthalmol Vis Sci, 2011). Based on this, we hypothesized that the iRECs would respond to DR stimuli with decreased junctional protein expression (excluding Claudin-2), reduced barrier properties, and impaired vascular network integrity. We initially investigated only iRECs as we wanted to demonstrate their ability to respond to disease triggers associated with a prominent retinal microvascular disease and thus their utility for modeling retinal specific pathologies. Incorporating iECs into this testing will provide a robust non-tissue specific control that will elucidate the retinal-specific phenotype and behavior of the iRECs.

We assessed both iRECs and iECs under DR conditions. iRECs exhibited pronounced decreases in branch length, network volume, and lumen diameter, while overall surface area remained unchanged, likely due to compensatory formation of smaller, pathological vessels (Fig. 2E). Junctional proteins ZO-1, CD31, VE-Cadherin, Claudin-5, and β -catenin were significantly reduced at cell-cell contacts, confirming barrier disruption, whereas Claudin-2 expression remained stable, consistent with *in vivo* observations (New Fig. S9A). UEA1 staining revealed glycocalyx degradation in iREC networks, further recapitulating DR pathology (Fig 2Eiii).

By contrast, iEC networks were less sensitive to DR insult, showing minor changes in junctional protein expression and network morphology (New Fig. S9B-C). These findings demonstrate that iRECs not only respond robustly to diabetic stress but also faithfully capture retinal-specific vascular dysfunction, highlighting their utility as a model for DR studies.

Fig. 2E. Quantification of iREC vascular morphology with or without DR treatment

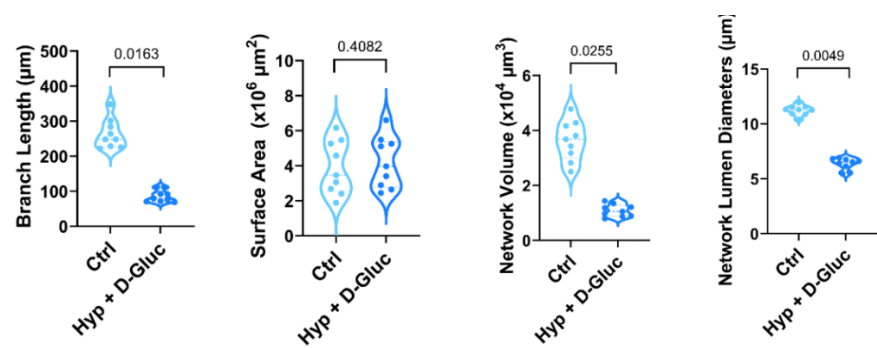


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment
Fig. 2Eiii. UEA statining in iREC vascular networks

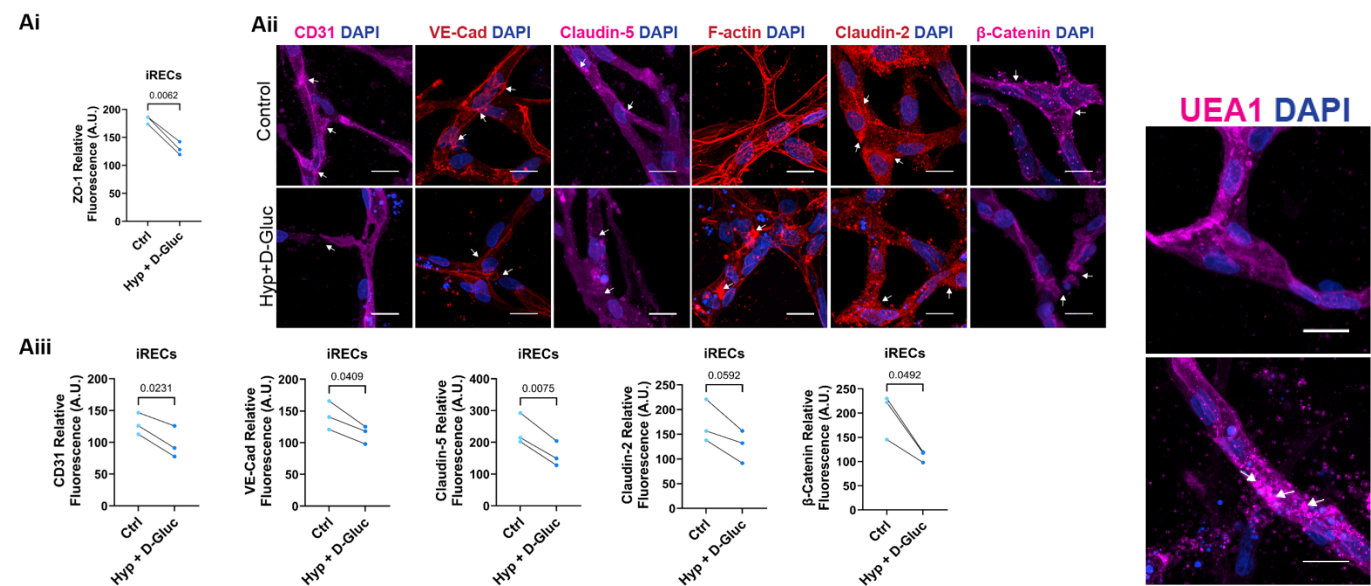
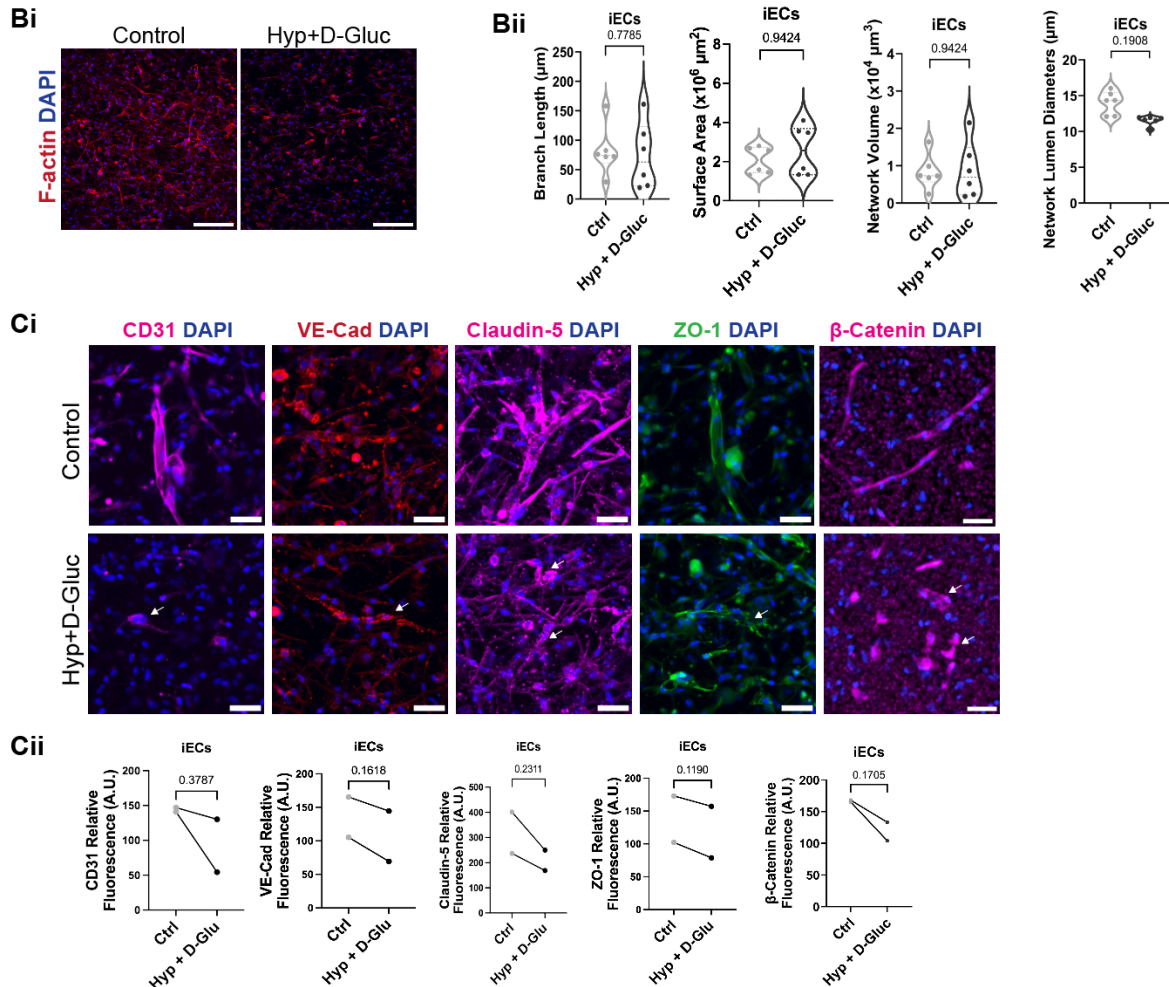


Fig. S9B. Quantification of iEC vascular morphology with or without DR treatment

Fig. S9C. 3D iEC vascular networks with or without DR treatment



9- It is not clear if that for the DR TEER assessment the cells were seeded in the same media, allowed to attach and form a monolayer and then subjected to DR model as there seem to be differences, albeit small, already at day 0.

We appreciate the reviewer's comment. We would like to clarify that both control iRECs and diabetic retinopathy-treated iRECs underwent the same experimental schedule, where they were seeded onto the Transwell on day 0 and received treatment (DR or control) on day 1. On day 0, both iEC and iRECs undergoing TEER assays were seeded on 24-well plate Transwell inserts containing $0.4 \mu\text{m}$ pores (Corning) and exposed to media composed of ECGM supplemented with $10 \mu\text{M}$ RepSox and 10% platelet poor human serum. Diabetic treatment was induced on day 1 and cells were exposed for 72-hours with media change daily (day 1 – day 4). We have updated the diabetic retinopathy treatment method section to clarify this. The first TEER measurement was made one hour after DR treatment was induced, so the slight variation in TEER values may be due to the acute impact of the DR conditions. Another possibility is that iRECs may exhibit a slower junctional maturation process compared to iECs, requiring more time to form a stable barrier. This difference in the kinetics of tight junction assembly could contribute to the minor variation observed at the early time point. Finally, The variability may also be due to the inherent variation characteristic of cells across technical replicates. Despite the apparent slight difference in TEER values on day one, post-hoc Tukey's analysis did not display any statistical difference at this timepoint.

10- Instead of comparing to iPSC-derived brain ECs, it would be more relevant to perform an unbiased scoring of the iECs and iRECs against DEGs in native RECs (which can also be obtained from published data, e.g. Menon et al Nat. Communications 2019; Watson et al Mol Vis 2020 etc.) to demonstrate which would score higher, without picking and choosing genes.

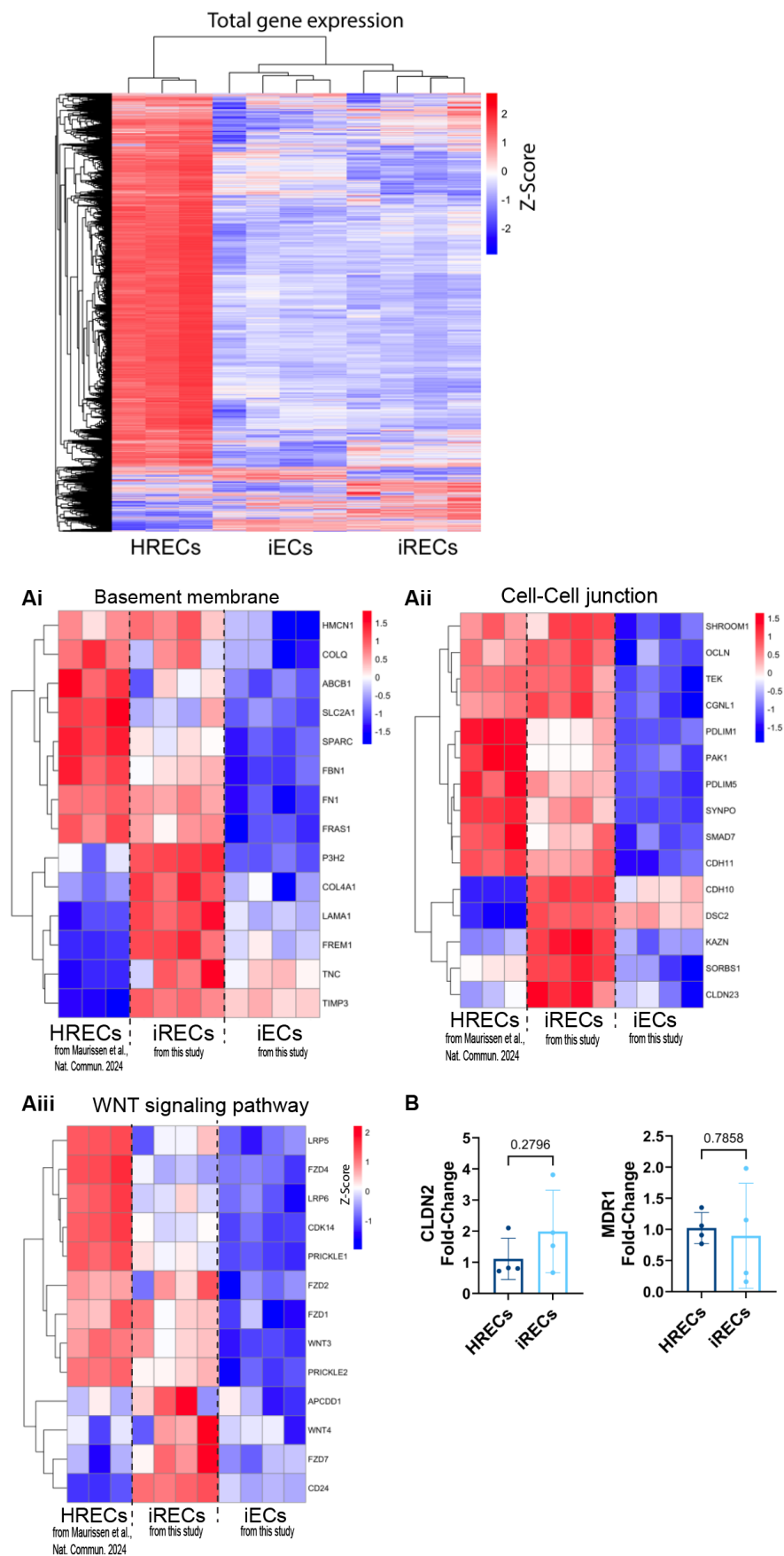
We thank the reviewer for this thoughtful suggestion. We would like to clarify that the genes used in our analysis were not selectively chosen but were derived through an unbiased approach. Specifically, we performed GO term enrichment analysis on the differentially expressed genes between iECs and iRECs to identify biologically relevant pathways. From the enriched GO categories, we then selected the top 20 most differentially expressed genes to highlight in the heat map. We better clarify this in the revised text.

We attempted to compare our data with the dataset from Menon et al. but found that it contained a limited number of retinal ECs (only 85 cells), limiting the reliability of the comparison. The dataset from Watson et al. was derived from bovine retina, which introduces species-specific differences that further complicate interpretation.

Group	Number of Cells
Rods	9145
Macroglia	5276
BPs	3155
RGCs	1514
ACs	497
Cones	211
HCs	146
Endo	85
Microglia	62

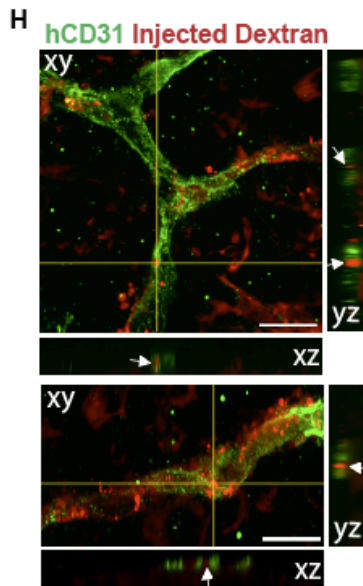
To directly assess retinal identity, we compared our iRECs to primary HRECs from Maurissen et al. (Nat Commun, 2024). Overall, iRECs display similarity to primary HRECs across multiple key gene sets relevant to retinal endothelial function (New Fig. S12A), confirming that iRECs recapitulate core transcriptional features of native retinal ECs. While the global gene expression patterns are not perfectly overlapping, this likely reflecting donor variability, culture conditions, and batch effect of RNA seq analysis.

To further substantiate these findings, we validated the retinal endothelial markers CLDN2 and MDR1 by RT-qPCR, which showed expression patterns consistent with HRECs (New Fig. S12B). Collectively, these results demonstrate that iRECs recapitulate the transcriptional and functional characteristics of human retinal ECs in a biologically meaningful way.



11- It is not clear from Fig 4C if iREC-derived vessels are perfused.

We thank the reviewer for this important comment. To verify whether the iREC-derived vessels are perfused, we administered 70 kDa rhodamine dextran via retro-orbital injection. We observed the perfusion of rhodamine dextran through human CD31 expressing iREC networks (New Fig 4H). This confirmed that the iRECs formed mature, perfusable vessels integrated with host vasculature.



12- “Corroborating these findings, a gene therapy study has reported the bilateral transfer of the substances between eyes, with visual improvement also observed in the contralateral, non-treated eye” – it would be great to have a bit more info on the ECs in the contralateral eye. Are they really cells?

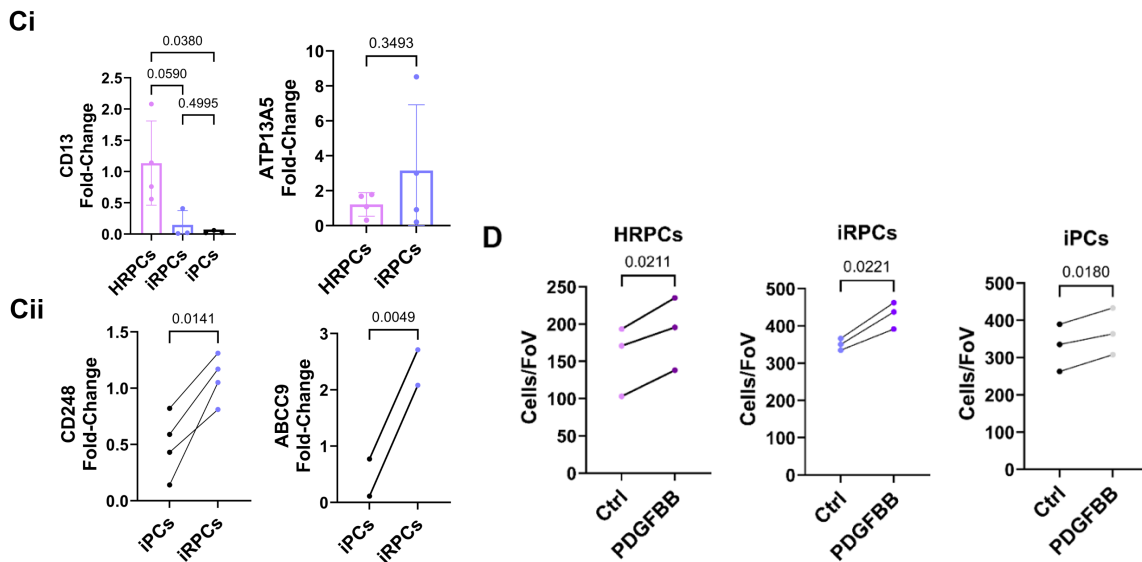
We thank the reviewer for this comment. The observation regarding potential bilateral effect was based on preliminary findings not directly related to the primary focus of this study. As our work centers on the regenerative capacity of iRECs within the treated retina, investigating whether ECs can migrate between eyes is beyond the current scope. Therefore, we have removed this statement from the manuscript to maintain focus and instead included this point in the limitations of the study.

13- Are these retina-specific pericytes or just pericytes? Very little validation is provided showing that these pericytes retina specific. Is the expression of PDGFb and Fzd4 enough to say they are retina-specific?

We thank the reviewer for this thoughtful comment. The molecular definition of retinal pericytes remains poorly established in the literature, making this a recognized limitation of the field. To evaluate the retinal specificity of our iRPCs, we compared them to human retinal pericytes (HRPCs) and non-tissue-specific iPSC-derived pericytes (iPCs) by RT-qPCR. iRPCs expressed comparable levels of the pan-pericyte marker CD13 (Fig. S16Ci) to HRPCs and iPCs, and similar levels of the retina-enriched gene ATP13A5 (Fig. S16Ci) to HRPCs. Importantly, iRPCs exhibited upregulated expression of retinal pericyte-associated markers CD248 and ABCC9 compared to iPCs (New Fig. S16Cii), suggesting a shift toward a retinal-specific pericyte identity.

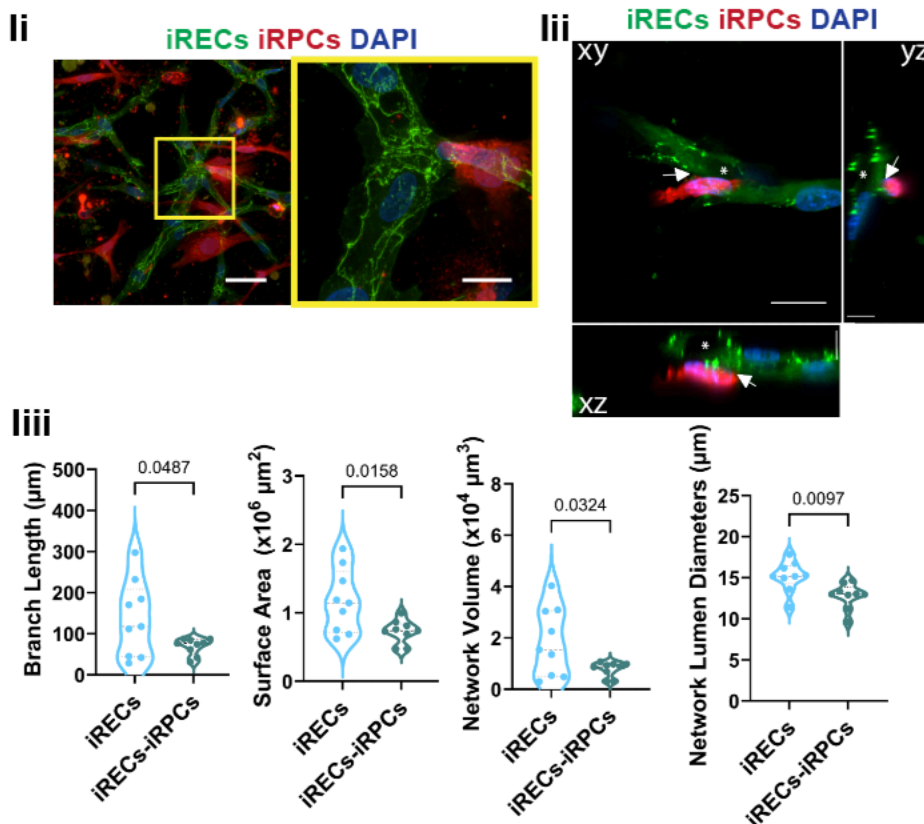
Functionally, iRPCs responded to PDGF-BB stimulation, a key endothelial-derived cue in iBRB development, through enhanced migratory capabilities within a Transwell assay. Importantly, their migration to the basolateral side of the Transwell was comparable to HRPCs and iPCs (New Fig. S16D), confirming their pericyte functionality.

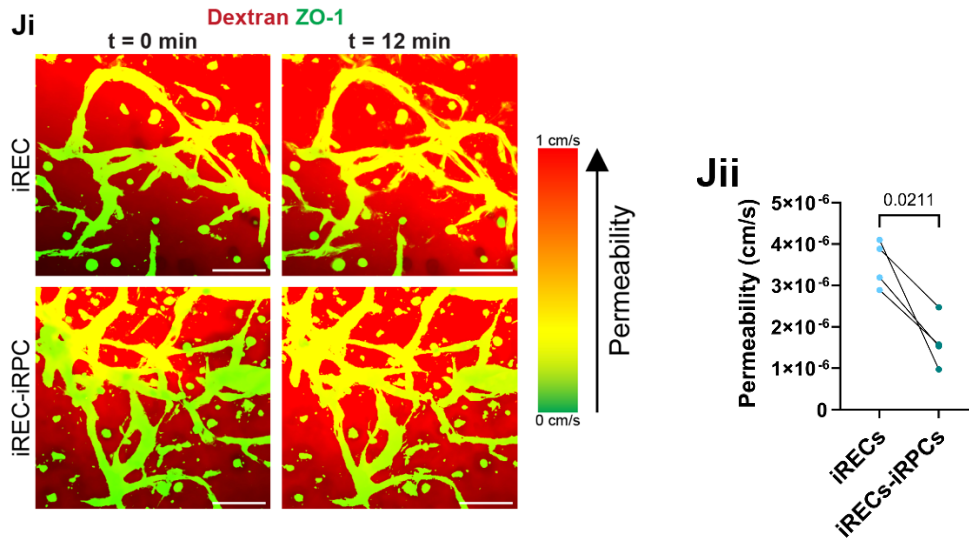
While a definitive molecular signature distinguishing retinal from non-retinal pericytes remains incomplete, our transcriptomic and functional evidence support that iRPCs acquire retina-enriched features. We acknowledge this remains an important limitation and have emphasized it in the *Limitations of the Study* section.



14- Does the inclusion of pericytes improve vessel formation, barrier function etc? This whole pericyte bit feels disconnected from the other parts of the manuscript. Perhaps using the model to study DR would be more relevant?

We thank the reviewer for bringing this to our attention. To establish the role of iRPCs within the MPS, we quantified network morphology and permeability. The presence of iRPCs within the model contributed to a decrease in vascular network branch length, surface area, volume, and lumen diameter (New **Fig. 5I**), recapitulating iBRB network morphology and the luminal size of human iBRB capillaries¹⁻⁵. The iRPCs also increased the barrier properties of the iREC networks, decreasing their 70 kDa rhodamine dextran permeability to a level consistent with *in vivo* permeability (New **Fig. 5J**).





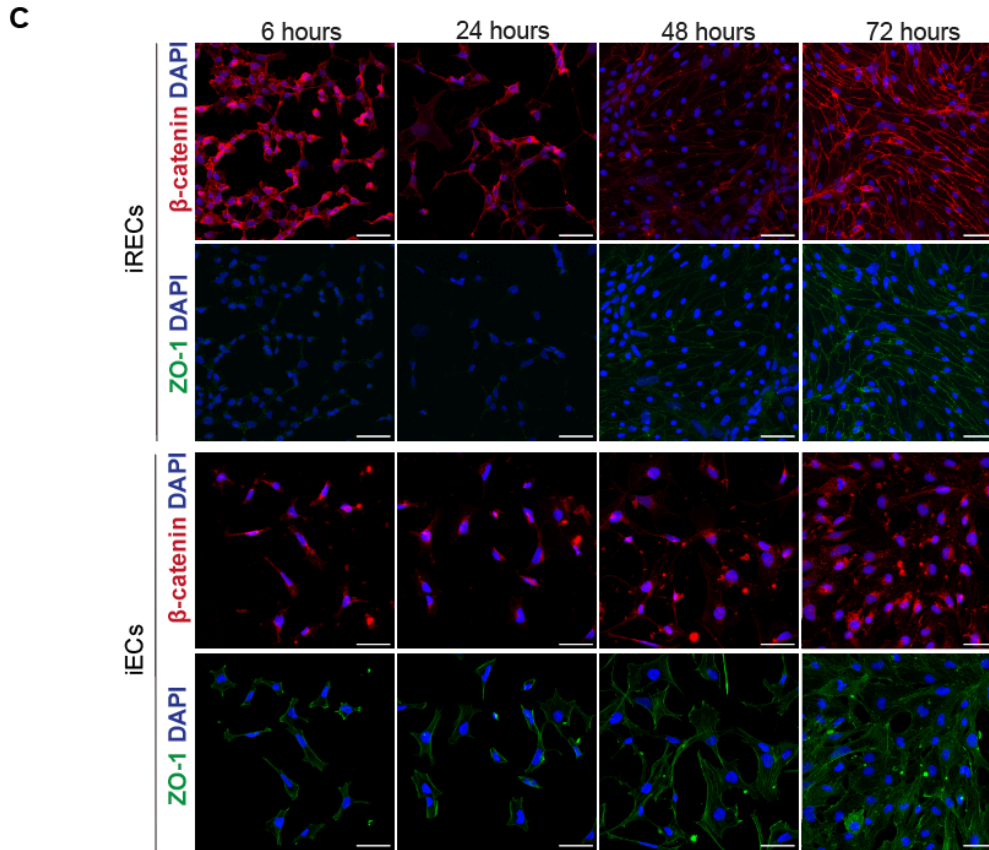
Minor criticism

1. Fig S1 caption reads: “and functional analysis” but there is no functional analysis reported.

We thank the reviewer for pointing this out. We have updated the caption of Fig S1 to remove “functional analysis”.

2. Panel C should also show generic ECs.

We thank the reviewer for this helpful comment. We have now included iECs in New Fig. S2C for direct comparison. We observed that iECs exhibited minimal β -catenin junctional localization, whereas in iRECs, substantial β -catenin translocated from the cytoplasm to cell–cell junctions within 48 hours after ETV2 induction. These findings demonstrate that Norrin-mediated Wnt/ β -catenin signaling is functionally activated during iREC specification, supporting the conclusion that iRECs recapitulate key aspects of retinal endothelial development and identity.



3. Fig S2 would benefit from quantitative analysis

We thank the reviewer for this suggestion. We have now included quantitative fluorescence analysis of the 3D vascular networks formed by iECs and iRECs (New Fig. S7). The results show significantly higher fluorescence intensity of junctional proteins ZO-1, Claudin-2, and Claudin-5 in iRECs, while canonical endothelial markers (CD31, VE-Cadherin, UEA1) remained comparable between the two cell types. These quantifications strengthen the conclusion that iRECs exhibit enhanced junctional organization consistent with a retinal-specific endothelial phenotype.

4. iBMEC – acronyms should be defined at first appearance

We thank the reviewer for pointing this out. The definition of iBMEC is now provided in the figure legend of New Fig. S13.

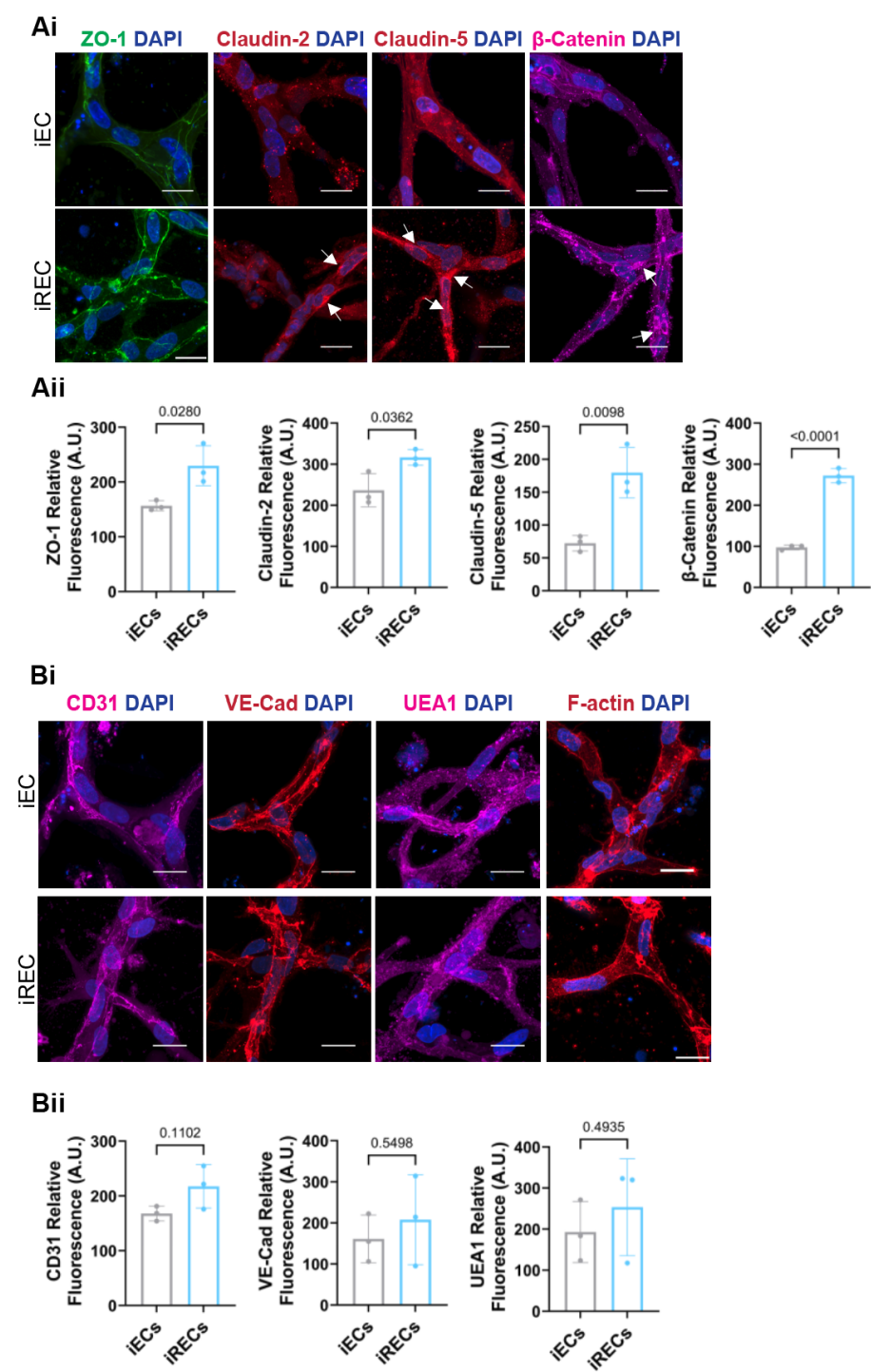
5. Fig 2 Ci could be moved to supplemental as it does not add critical info

“Specifically, the iREC networks contain heightened membrane-bound Claudin-2 and Claudin-5 cell-cell contact expression relative to iEC networks (Fig S7A)” there is no quantification to allow this statement. Claudin -5 to me looks the same. Providing only 1 image without quantification demonstrate lack of rigor.

We thank the reviewer for this valuable comment. We have updated the figure (New Fig. S7) to provide more accurate representative figures and include quantitative analysis of fluorescence intensity for junctional markers, and revised the text accordingly.

The iREC networks exhibited higher overall fluorescence intensity of ZO-1, Claudin-2, Claudin-5, and β -catenin compared to iEC networks (New Fig. S7A), consistent with their more continuous junctional distribution observed in immunostaining. In contrast, expression of canonical endothelial markers (CD31, VE-Cadherin,

UEA1) were comparable between iREC and iEC networks (New Fig. S7B), confirming that both maintain endothelial identity.



6. Provide lower magnification images for Fig 5 Dii and assessment of network perfusion

We thank the reviewer for the suggestion. Lower magnification images have been added as supplementary Figure 15Eii.

7. Missing or unclear details about statistics, protocols or materials

We thank the reviewer for pointing this out. We have updated the method section to include additional details on protocols, cell numbers, experimental timeline, statistics, and materials used.

8. For quantifications (e.g. Fig 2Dii) authors need to provide details of what was assessed, were all points measured from the same assays or 8 different biological replicates (differentiations and different runs)? In other words, are these technical replicates of real separate assays?

We thank the reviewer for bringing this to our attention. The quantification in Fig 2Dii was performed using three independent biological replicates, each containing three technical replicates (total of nine data points shown in the graph). For each technical replicate, ten cells were quantified to obtain the fluorescence intensity values for the entire sample. We included all the datapoints within the figure to enable easy visualization of the results. We have updated figure legends and the methods section to clarify our quantification. We have updated figure legends and the methods section to clarify our quantification for all experiments.

Reviewer #2 (Report for the authors (Required)):

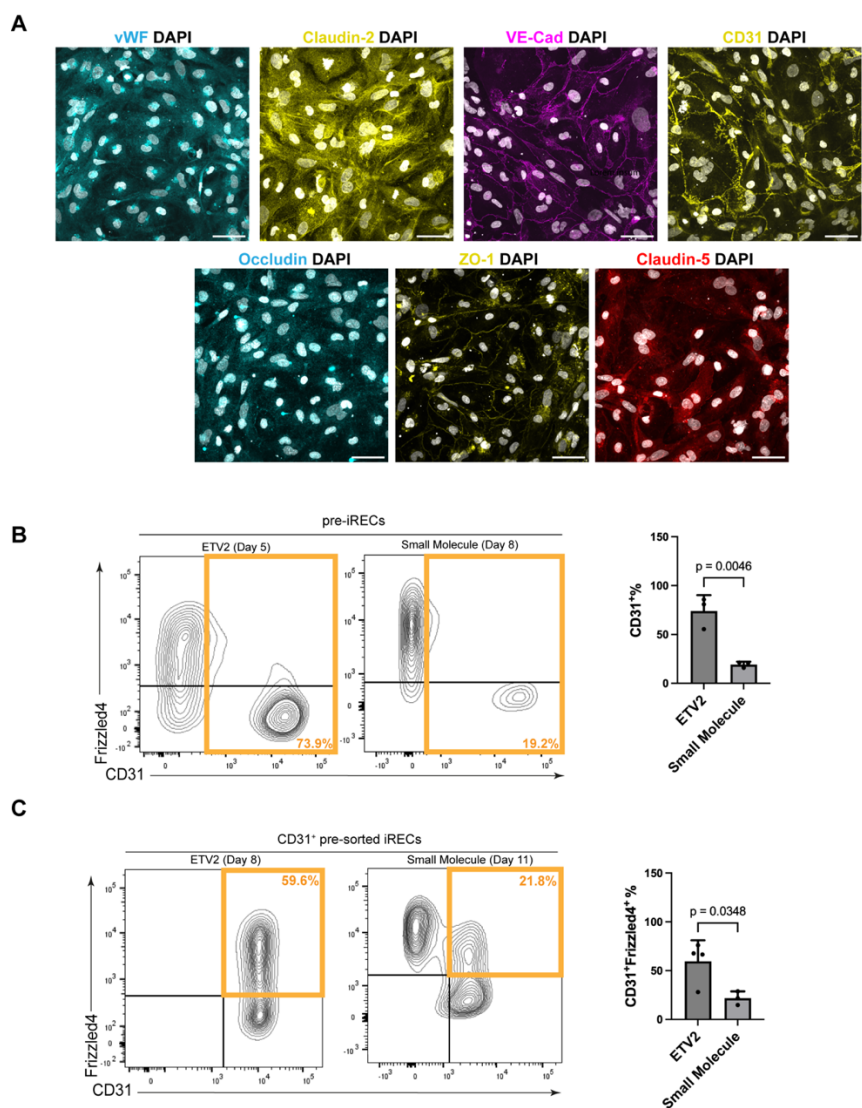
The reviewed paper aims to modify the existing differentiation protocol of endothelial cells (ECs) and pericytes (PCs) from iPSCs to mimic the retinal EC and PC phenotypes. Although the proposed project possesses high impact due to providing materials for otherwise hardly accessible cell types for disease modeling and therapeutic approaches, the current state of the project cannot yet be considered complete.

(1) The authors modified the existing protocol for electroporation-induced EC differentiation to drive additionally treating with Norrin and vitronectin to induce the retinal EC (iRECs). As a result the cell population iREC positive for CD31 and Fz4 was received after two rounds of cell sorting. For the iREC induction protocol, I have the following questions:

Why was the electroporation protocol chosen as a base protocol? Did the authors try standard EC induction with growth factors and it didn't work or was the electroporation protocol the first choice? Electroporation has limitations for possible therapeutic usage: although a non-integrative method was used, it still increases the risk for genomic instability.

We thank the reviewer for this important point. We evaluated the iREC small-molecule differentiation protocol (New Fig. S5) and found that, while it successfully generated iREC expressing functional iREC markers, the overall efficiency and yield of CD31⁺ cells were significantly lower compared to the ETV2 electroporation-based method (19.2% vs. 73.9%). Specifically, the CD31⁺Fz4⁺ iREC population pre-Fz4 sorting was significantly reduced using the small molecule approach (21.8% vs. 59.6%). Based on these findings, we selected the ETV2 electroporation protocol as our base method to ensure robust production of iRECs. ETV2-based protocols have generated biologically accurate ECs^{6,7}, supporting our utilization of this technique.

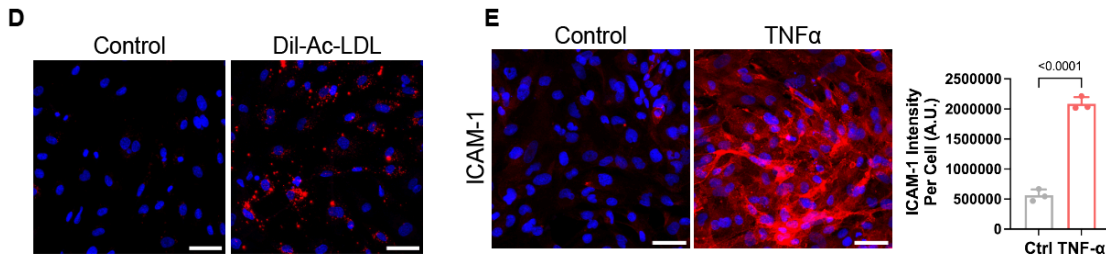
We acknowledge that electroporation may increase the risk of genomic instability, which is an important consideration for therapeutic applications. In this study, electroporation was selected for its efficiency and reproducibility in generating high yields of CD31⁺Fz4⁺ iRECs population. At the same time, alternative strategies for ETV2 induction have been reported, including stable doxycycline-inducible iPSC lines (Gong et. al., CSC, 2025), and viral-mediated delivery (Morita et. al., PNAS, 2014). An emerging mRNA delivery technology based on mechanical disruption (Stewart and Sharei et. al., Nature, 2016) represents a promising alternative to induce transient gene expression with reduced genomic risk. This approach could be further explored in future studies to improve the clinical applicability of iREC generation.



Were there any functional assays done to prove the EC differentiation? Several previous attempts to receive brain ECs phenotype resulted instead in the differentiation of epithelial-like cells then ECs. Therefore it is essential to verify the EC identity of iRECs with functional assays, such as (1) an actively take-up acetylated LDL and (2) ICAM expression upon TNF- α treatment. These additional experiments would confirm that ECs preserved cell identity after Norrin and vitronectin induction.

We thank the reviewer for this insightful comment. We have confirmed the endothelial identity of iRECs through immunofluorescence imaging and flow cytometry which showed robust expression of endothelial markers including CD31 (Fig. S2A, and Fig. 1Ci-iv), vWF (Fig. 1D), and VE-cadherin (Fig. 1D). In addition, iRECs demonstrated endothelial functionality by forming 3D lumen-like structures with comparable branch length and surface area to iECs in 3D collagen type-I hydrogels (Fig. 2Ci) and within the iBRB-on-chip system (New Fig S15Bii and Ciii).

Furthermore, iRECs actively internalized Dil-acetylated LDL and responded to TNF α stimulation with increased ICAM-1 expression (New Fig. S2D-E), confirming that they maintain hallmark endothelial functions.



(2) The authors used the expression of two cell markers, CD31 and Fz4, as hallmarks of retinal EC and PC specification. However, both iECs and pre-iRECs were shown to express CD31 and Fz4. iREC induction was performed by adding the Fz4 ligand, Norrin. The efficiency of iREC differentiation was assessed by the increase in the Fz4⁺ cell population. But isn't the most obvious explanation for this increase a positive feedback loop, where Norrin induces the expression of its receptor, Fz4? If so, the results may reflect Norrin's effect on ECs rather than the emergence of a distinct iREC population.

We thank the reviewer for raising this important point. While it ligand exposure could potentially influence receptor expression, to our knowledge, there is no literature evidence supporting a positive feedback loop between Norrin and Fz4.

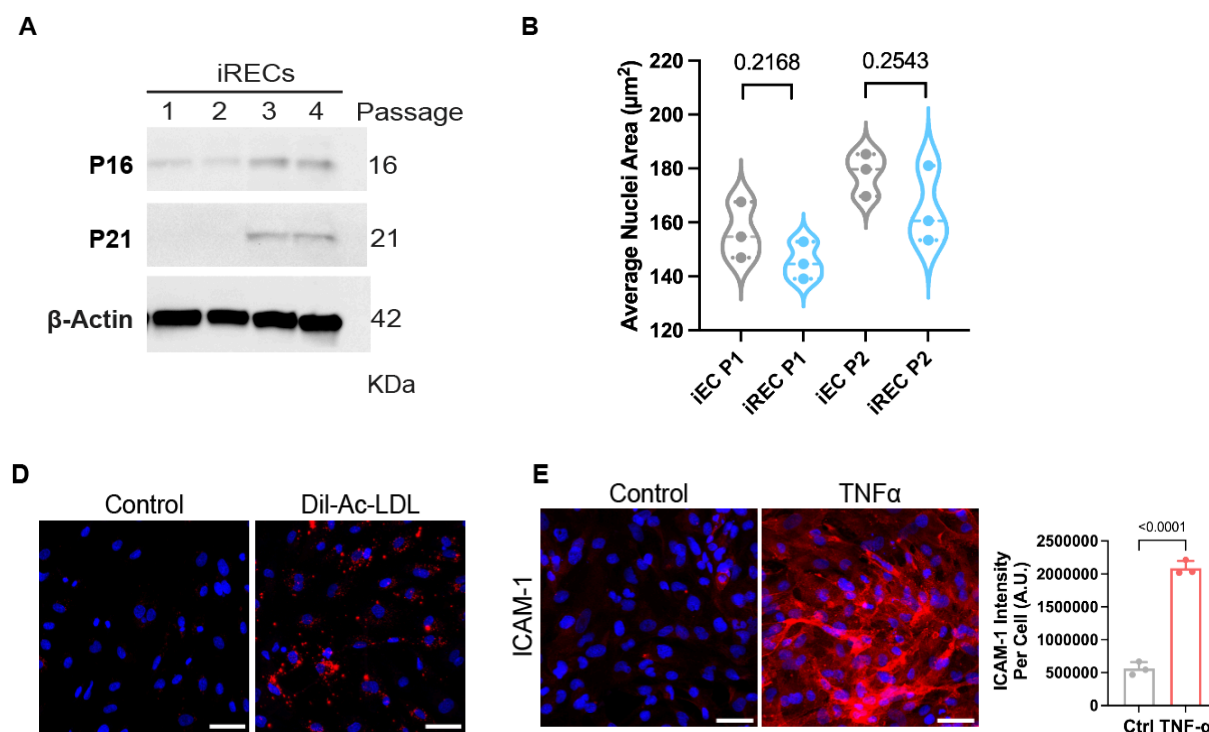
The CD31⁺ pre-iRECs were cultured in the absence of Norrin from day 5 to day 8, using endothelial cell growth medium (ECGM) supplemented with 10 μ M RepSox and 10% platelet poor human serum. On day 5, 58.3% of CD31⁺ pre-iREC expressed Fz4, but this portion increased to 66.5% by day 8, despite the removal of Norrin. This suggests that the increase in Fz4 expression is not likely a direct result of ongoing ligand stimulation rather a progression in cell identity toward an iREC phenotype.

Furthermore, iRECs exhibit hallmark retinal endothelial characteristics, including higher TEER and active GLUT1-mediated glucose transport (Fig. 2A – B), and display a distinct transcriptomic profile from iECs (Fig. 3), confirming that they represent a specialized retinal EC population rather than a Norrin-stimulated iEC variant.

(3) Figure 1D demonstrates the differences in morphology and marker expression of iECs and iREC. The morphology of iECs appears healthier: cells are uniformly shaped and properly aligned with each other. The round and irregular shape of iRECs, on the other hand, reminds features of senescence or dysfunctional ECs.

We thank the reviewer for this insightful comment. To address concerns regarding potential senescence, we performed western blot analysis for cyclin-dependent kinase inhibitors p21 and p16 that induce senescent cell-cycle arrest across multiple iREC passages (New Fig. S4A). Senescence markers was upregulated beginning at passage 3 and therefore all experiments were conducted using passage 1-2 iRECs, where no significant upregulation of senescence markers was detected. We also compared the nuclear area of iECs and iRECs between passages 1-2 and found no significant difference (New Fig. S4B). This suggest that the distinct morphology and marker expression of iRECs likely reflects inherent phenotypic difference from iECs rather than senescence-associated changes.

To further verify that iRECs are functional ECs, we conducted functional assays and found that iRECs actively internalized Dil-acetylated LDL and responded to TNF α stimulation with increased ICAM-1 expression (New Fig. S2D-E), demonstrating that they are mature, functional ECs.

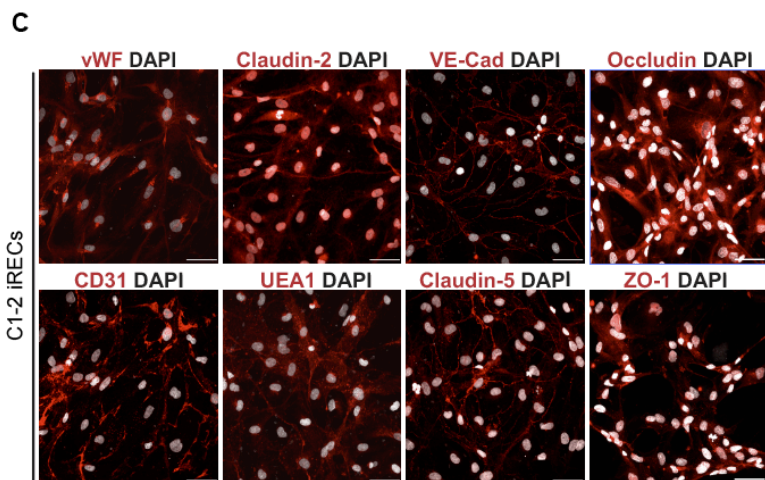


(4) Figure S2 demonstrates the staining and morphology of HRECs. The cell density appears low and cell morphology is generally unhealthy. Authors should consider excluding these cells from the analysis since results received with unhealthy cells are unreliable.

We thank the reviewer for this insightful comment. We obtained primary HRECs directly from Cell Systems and used them only between passage 4-6. Within these passages we did not observe the loss of endothelial marker expression or significant morphological abnormalities that would suggest compromised cell health. Moreover, HRECs from the same supplier and within comparable passage ranges were used in our previous study (Cho and Macklin et al., JCI Insight, 2020), where they exhibited similar morphology and EC marker expression. Based on these considerations, we are confident that the HRECs used in this study were healthy and suitable for analysis.

(5) Figure S2C should prove the robustness of the protocol by using another hiPSC line to differentiate iRECs. However, the authors do not show the expression of ZO-1 and Occludin, two of the cell junction markers, they claimed to be increased by this protocol compared to iECs.

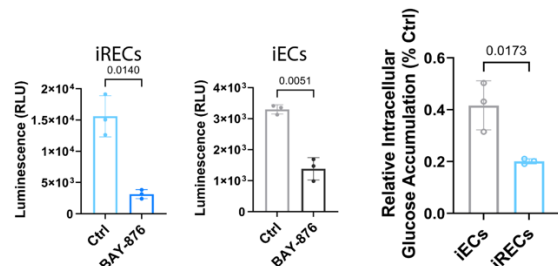
We thank the reviewer for this comment. We have included immunofluorescence staining of ZO-1 and Occludin for C1-2 iRECs.



(6) Authors demonstrate that iRECs have a higher TEER than iECs. However, they do not provide the data on Bay-876 treatment. Bay-876 treatment significantly reduces the glucose uptake due to GLUT1 inhibition of iRECs, but does it have the same effect on iECs?

It is crucial to assess the GLUT1 transport in both cell types if the glucose transport serves as a retinal endothelial phenotype.

We thank the reviewer for this important comment. We have now included iECs in the GLUT1 transport assay (New Fig. S6C). GLUT1 is the primary facilitator of glucose transport, a key feature of retinal endothelial cells. At baseline, iRECs exhibited ~5-fold higher glucose uptake than iECs (1.5×10^4 vs. 3×10^3 ; Fig. 2B, New Fig. S6Ci). Treatment with the selective GLUT1 inhibitor Bay-876 reduced glucose uptake by 80% in iRECs but only 60% in iECs (New Fig. S6Cii). These results demonstrate that iRECs possess higher GLUT1 activity and transporter functionality, reinforcing their retinal-specific metabolic phenotype.



(7) The authors claim in Figure S3A, that iRECs show increased Claudin-2 expression at cell-cell junctions compared to iECs. Later, in Figure S4C, they claim that the Claudin-2 expression of iRECs in diabetic conditions does not differ from the control. However, the shown representative images of Claudin-2 staining in iECs and diabetic iRECs look very similar. So either there is no Claudin-2 expression difference between iECs and iRECs, or the diabetic treatment of iRECs caused a reduced localization of Claudin-2 at cell-cell junctions.

We appreciate the reviewer's comment. To address this concern, we provided more accurate representative figures and quantified the total fluorescence intensity of Claudin-2 expression in both iEC and iREC vascular networks (New Fig. S7A). This analysis confirmed that iRECs exhibit higher Claudin-2 expression compared to iECs. Similarly, we provided new representative figures for iRECs treated with DR and quantified their Claudin-2 protein expression (New Fig. S9Aii-iii). Claudin-2 expression was consistent between iRECs treated with and without DR condition.

Fig. S7A. 3D iEC and iREC vascular networks

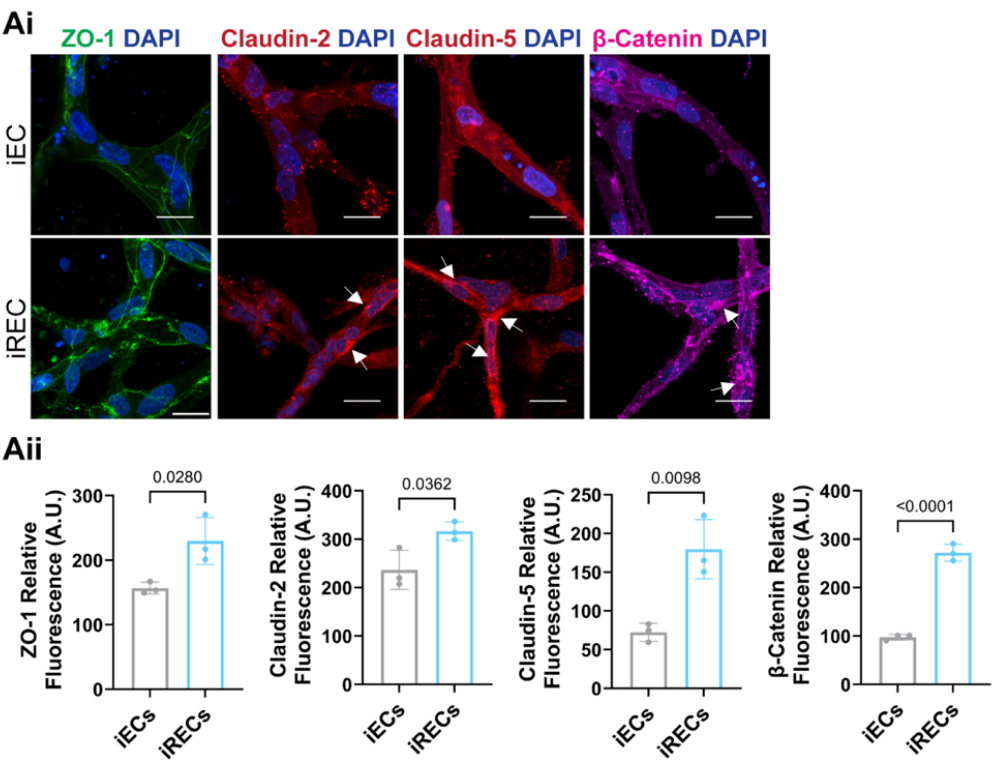


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment

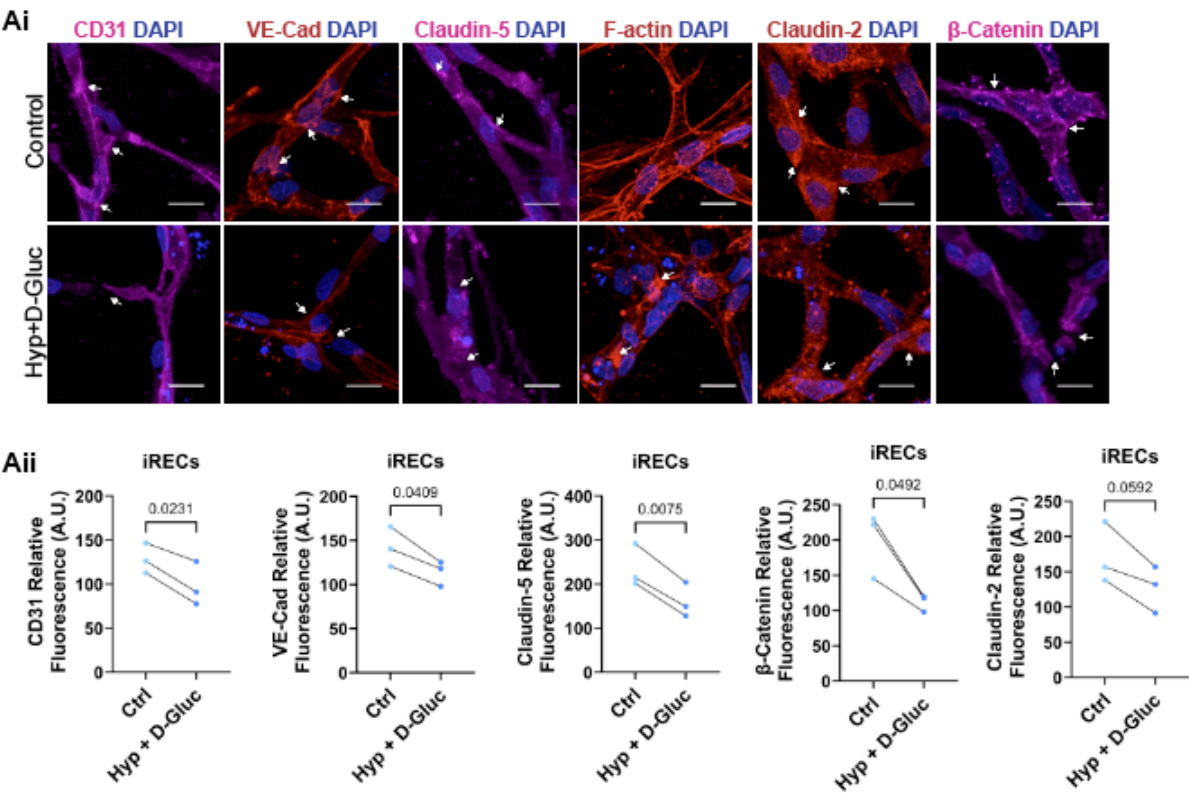
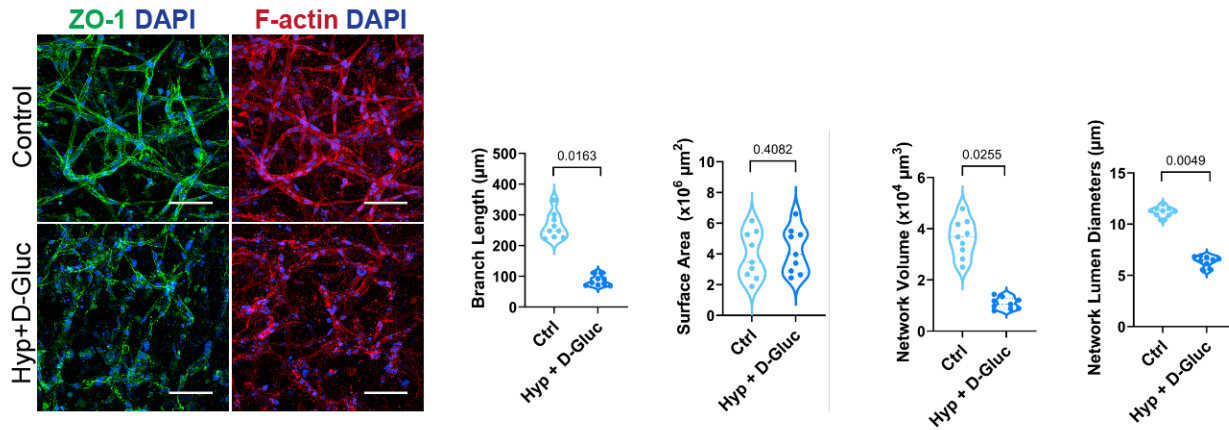


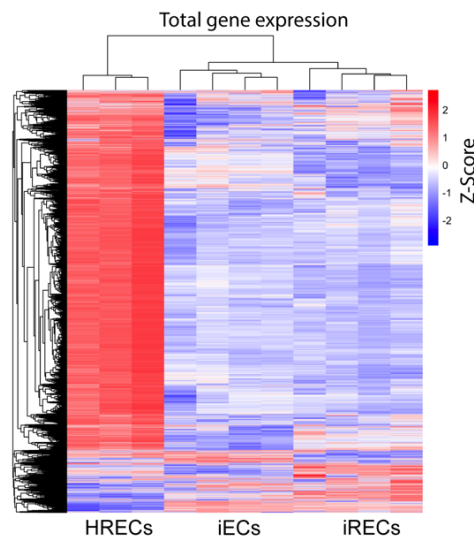
Fig. 2E. 3D iREC vascular networks with or without diabetic treatment and morphology quantification

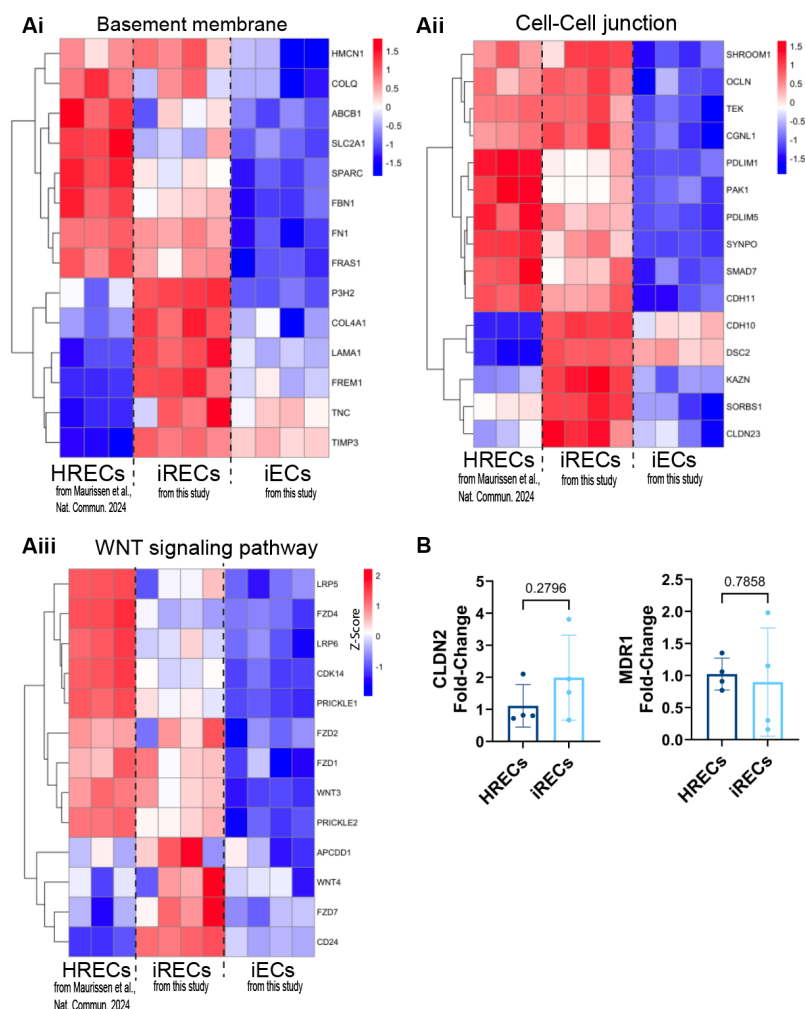


(8) The authors do a transcriptomic analysis of iRECs, and iECs and compare them with iPSC-derived brain ECs. Of course, there are similarities (both ECs) and differences (different differentiation protocols). Would it not have been more meaningful to compare the iRECs and iECs with human retinal endothelial cells, since the focus of the paper is to prove that iRECs show more retinal identity than iECs?

We thank the reviewer for this important comment. We initially did not compare iRECs with Primary HRECs due to concerns that the primary cells lost tissue-specific phenotypes when cultured *ex vivo*. As a result, we originally compared the iRECs to iECs as they have been previously validated and their robustness has been confirmed.

To address reviewers suggestion, we compared our samples to primary HRECs from Maurissen et al. (Nat Commun, 2024). Importantly, we observed similar expression between our iRECs and human primary retinal ECs across multiple relevant gene sets (New Fig. S12A). While the global gene expression heat map does not show perfect similarity, this may partly reflect the inherent variability among primary cell donors, differences in culture conditions, or batch-related confounding factors. These limitations highlight the need of iPSC-derived retina ECs as a more consistent and reproducible model. To further strengthen this comparison, we performed RT-qPCR validation of the retinal endothelial markers, CLDN2 and MDR1, confirming iREC expression patterns similar to HRECs (New Fig. S12B).





(9) Revascularization experiments in the ischemic eye were conducted only with iRECs. To demonstrate the increased therapeutic potential of iRECs, it is important to compare them with iECs. Ideally, the therapeutic effect of iRECs should also be compared with that of primary mouse retinal endothelial cells.

We thank the reviewer for this comment. To directly compare therapeutic efficacy, we included iECs as a control in the OIR model. Consistent with prior reports, iEC treatment reduced neovascularization (NV) and vaso-obliteration (VO) relative to PBS controls (New Fig. S14A). Both iECs and iRECs integrated with host vasculature and reduced vascular leakage (New Fig. S14B, S14F, 4C, 4G). Functional perfusion of iREC-derived lumen-like structures with rhodamine-dextran confirmed successful vascular integration and maturation (New Fig. 4H). Notably, iRECs demonstrated better efficacy than iECs, restoring physiological vascular network with smaller segment volumes and lumen diameters while iECs did not impact these parameters (Fig. 4E and New Fig. S14D). These results emphasize the therapeutic and biological significance of retinal-specific ECs.

Primary mouse retinal ECs were not included due to species-specific differences that could confound interpretation. Comparison with non-retinal iECs provides a relevant benchmark, highlighting the enhanced retina-specific reparative potential of iRECs.

Fig. S14A-B. iECs injected OIR retina

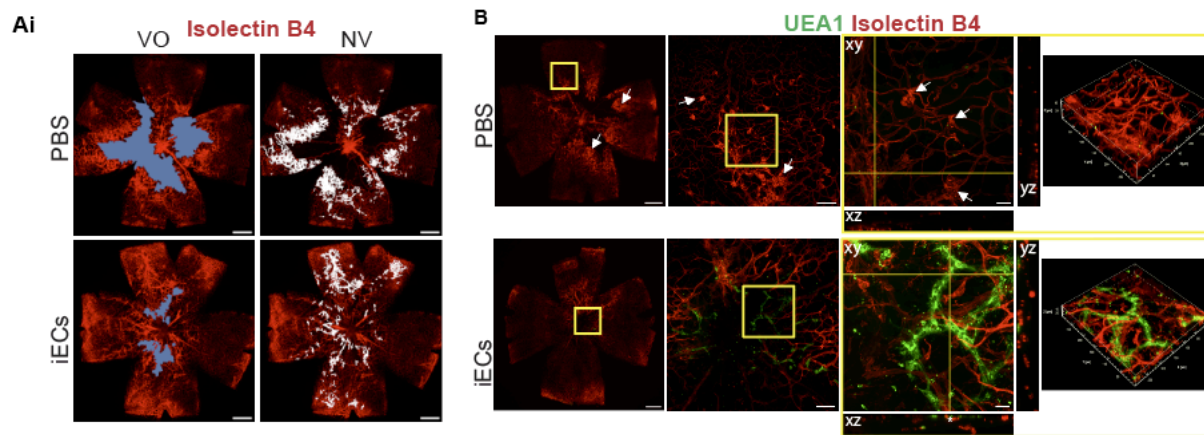


Fig 4E. Quantification of mouse vascular network segment volume and lumen diameter across PBS and iREC injected groups

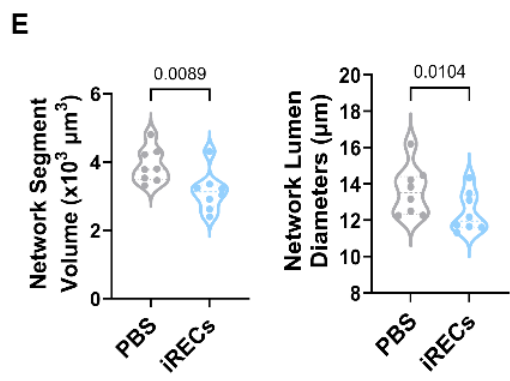


Fig. S14F. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iEC injected OIR retinas

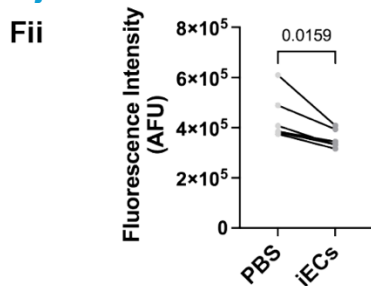


Fig.4C iRECs injected OIR retina.

Fig.4Gii. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iREC injected OIR retinas.

Fig. 4H. iREC networks perfused by injected 70 kDa rhodamine dextran within OIR retinas

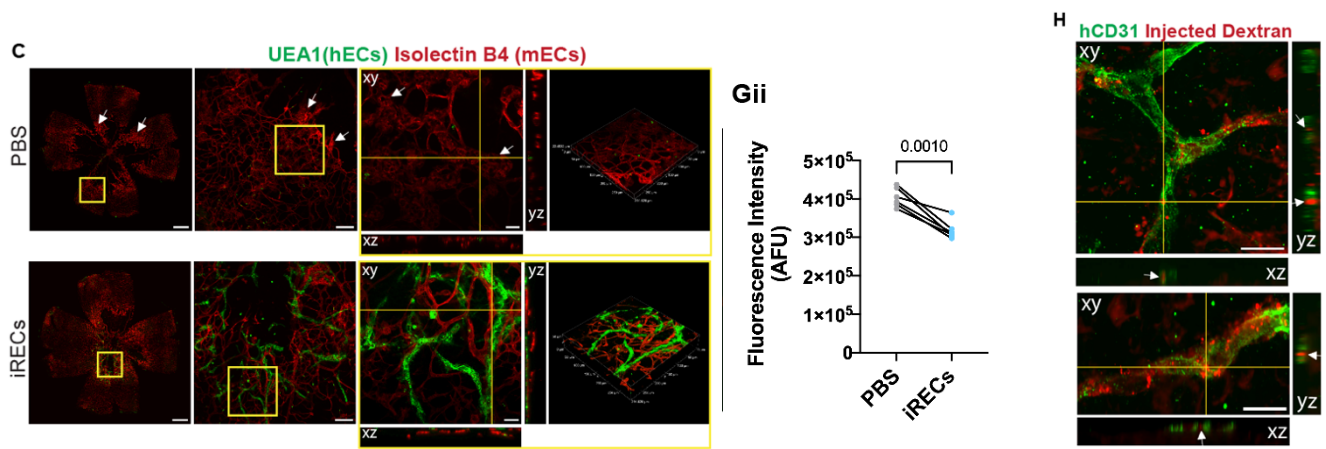
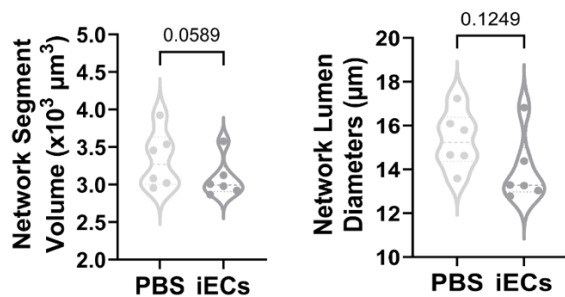
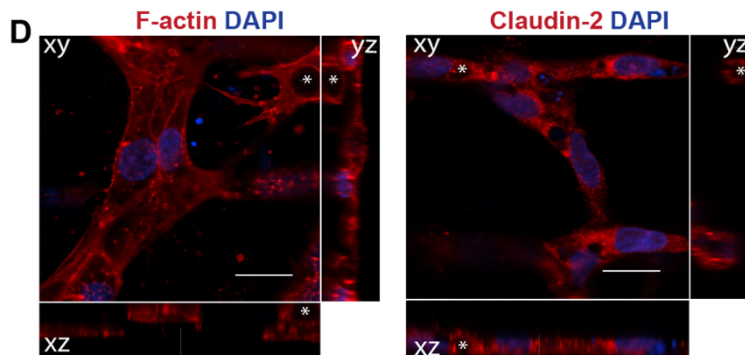


Fig 4E. Quantification of mouse vascular network segment volume and lumen diameter across PBS and iEC injected groups



(10) In Figure 5Di, the authors show the existence of a lumen in the vasculature. However it seems, that not every asterisk is indeed a lumen, some might be just holes in the vessels (especially the ones shown in the xy-image in which you can not prove that the lumen is enclosed by ECs).

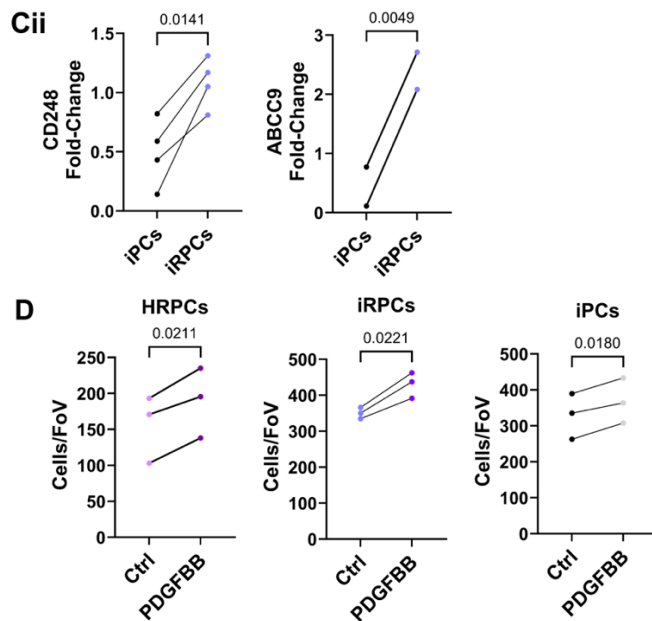
We thank the reviewer for pointing this out. We have revised Fig. 5D to ensure that asterisks indicate only lumens enclosed by ECs. We specifically selected lumens that are supported by ECs that can be visualized in the xy-, yz-, and/or xz-plane(s).



(11) Are there any differences in marker expression between mesodermal PCs and iRPCs? The effect of increased Fz4 could be the same as in iRECs: the introduction of the ligand Norrin may upregulate Fz4 expression through a positive feedback loop, rather than proving the existence of a new cell subpopulation.

We thank the reviewer for this insightful comment. iRPCs were exposed to Norrin only during the six day differentiation phase and subsequently cultured in Pericyte Medium without Norrin from passage 1 through passage 10. This long-term maintenance without Norrin indicates that the sustained FZD4 expression reflects stable retinal pericyte specification rather than a transient positive feedback effect.

To further substantiate retinal identity, we performed qPCR analysis of retinal pericyte-specific markers, CD248 and ABCC9, which showed significantly higher expression in iRPCs compared to non-retinal iPCs (New Fig. S16C). Functionally, iRPCs demonstrated robust migration in response to PDGF-BB, confirming canonical pericyte competence (New Fig. S16D). While we acknowledge that retinal pericyte characterization remains limited in the literature, these results provide strong evidence of iRPC lineage fidelity and functional capacity, as discussed in the “Limitations of the study” section.



(12) The Permeability assay is missing in the methods.

We thank the reviewer for pointing this out. We have updated the methods section to include a detailed description of the permeability assay.

(13) The used iPSC line contains EGFP-tagged ZO-1. However, the images are still referred to as immunofluorescence of ZO-1. Were these cells additionally stained with antibodies against ZO-1?

We thank the reviewer for pointing this out. The hiPSC line used was the mEGFP-tagged TJP1 WTC line obtained from the Coriell Institute. We did not stain these cells with an additional antibody against ZO-1. The only cells we stained for ZO-1 utilizing a primary antibody was the C1-2 hiPSC and primary HREC lines as they were not tagged. We have updated ZO-1 to ZO-1-GFP to reflect the EGFP-tagged signal in the figure legends throughout the manuscript.

In summary, several critical issues need to be addressed before the paper can be considered for publication. The presented results might be interpreted as the Norrin impact on gene expression, TEER, and microvasculature formation, but they are not proof of successful iREC/PC differentiation protocol. Due to multiple methodological inconsistencies and a lack of results on multiple cell lines, I can not recommend the paper for publication.

We thank the reviewer for their thoughtful comments and recognition of the potential impact of our work. We have addressed all the concerns raised with new data that strengthen the rigor, clarity, and novelty of our findings.

Reviewer #3 (Report for the authors (Required)):

This manuscript describes a method to derive human retinal endothelial cells (iRECs) from hiPSCs by activating the Wnt/ β -catenin signaling pathway, specifically through Norrin/Fz4 signaling. The authors claim that these iRECs recapitulate key molecular, phenotypic, and functional features of native retinal endothelial cells, including barrier function, responsiveness to diabetic retinopathy (DR)-like conditions, and in vivo vasculogenic potential in an oxygen-induced retinopathy (OIR) model. They further demonstrate the integration of iRECs with iPSC-derived retinal pericytes to form a perfusable, microphysiological iBRB-on-a-chip platform. This work is positioned within the fields of vascular biology, regenerative medicine, and ophthalmology, where robust, tissue-specific endothelial cell models are critical for disease modeling and therapeutic development.

The development of protocols aimed at generating endothelial cells with bona fide tissue-specific identities is an area of growing importance in vascular biology, with significant implications for both mechanistic studies and translational applications. In this context, the authors' efforts to define and validate a retinal-specific endothelial phenotype from hiPSCs address a timely and relevant question. The manuscript presents a technically ambitious and well-structured body of work, and the integration of iRECs into a microphysiological model is a notable strength.

However, based on the current data, the study does not yet establish that iRECs have acquired a definitive retina-specific identity that functionally distinguishes them from other iPSC-derived endothelial cells. Key aspects of tissue specificity—particularly at the transcriptomic, functional, and in vivo levels—would benefit from further support.

I have the following major concerns.

Major Concerns

1. The study does not provide an adequate dissection of the differentiation protocol components. The iREC protocol relies on sequential exposure to Norrin and vitronectin (Days 2–5), followed by RepSox (Days 5–8), but the rationale for this timing and factor selection is not well explained. It is unclear whether these cues act through their anticipated signaling pathways or how each contributes to the final phenotype. For example, it would be informative to compare iECs generated without Norrin and vitronectin but with RepSox, or vice versa. Without these controls, it is difficult to determine whether all three factors are necessary or if one alone is sufficient.

We thank the reviewer for this insightful comment. Norrin and vitronectin were included in the early differentiation phase immediately following mesodermal induction to promote retinal EC specification. Successful endothelial differentiation was confirmed on day five by robust CD31 expression. RepSox was incorporated during the later maturation phase to enhance barrier function, consistent with its reported role in supporting retinal EC maturation. In our additional experiment, iECs cultured without Norrin and vitronectin possessed minimal β -catenin activation and junctional localization and failed to show Fz4 endocytosis (New Fig. S1C and S1D). As the iECs represent a negative control for the iRECs treated with Norrin and vitronectin, these results confirm that Norrin and vitronectin induce a retinal-specific EC signature and phenotype that is absent from non-tissue specific iPSC-derived ECs.

To validate the temporal contributions of these factors, we performed RT-qPCR at day five with or without Norrin and vitronectin supplementation (New Fig. S1E). The iRECs differentiated without Norrin and vitronectin had decreased expression of the retinal EC marker, FZD4, compared to control. Their expression of the retinal EC-associated genes, CLDN10, CLDN23, CLDN2, CLDN5, and IGF2 expression was also trending toward a decrease relative to control, demonstrating the essential contributions of Norrin and vitronectin to retinal EC development and maturation. We further evaluated the role of RepSox in barrier development using TEER and permeability assays. iRECs cultured without RepSox exhibited decreased TEER and impaired barrier function over four days, whereas RepSox supplementation significantly enhanced barrier integrity and reduced permeability (New Fig. S3A-B). These results demonstrate that all three factors (Norrin, vitronectin, and RepSox) are essential to deriving mature and functional retinal-specific ECs

Fig. S1C. Expression of β -catenin and ZO-1 proteins on pre-iRECs and iECs

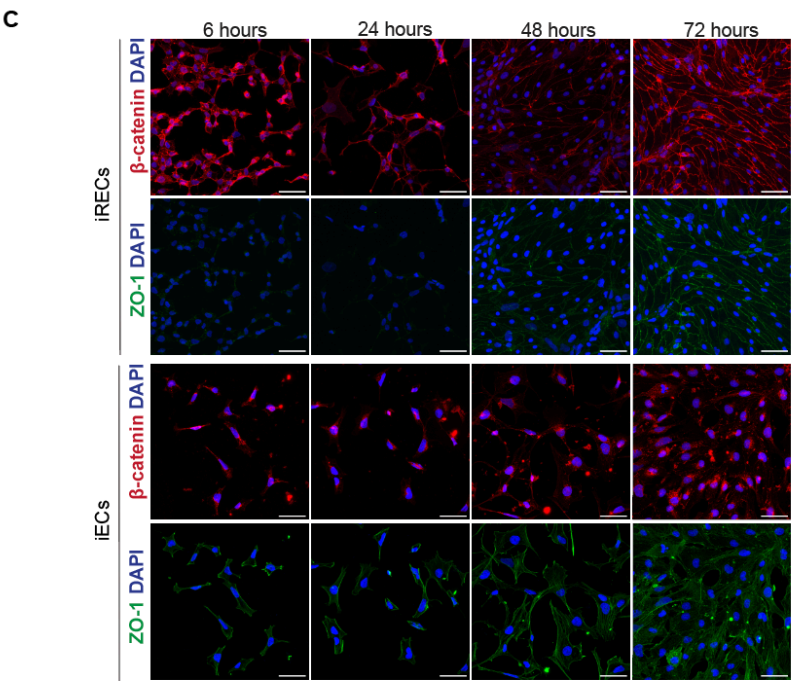


Fig. S1D. Flow cytometry analysis of iECs

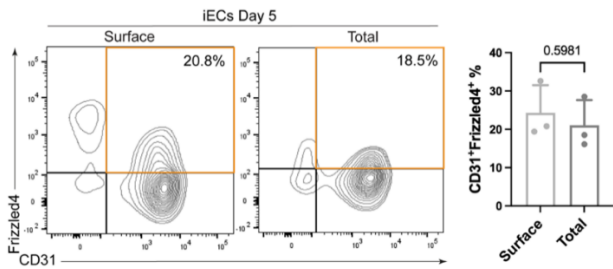


Fig. S1E qRT-PCR analysis of iRECs treated with or without Norrin/vitronectin

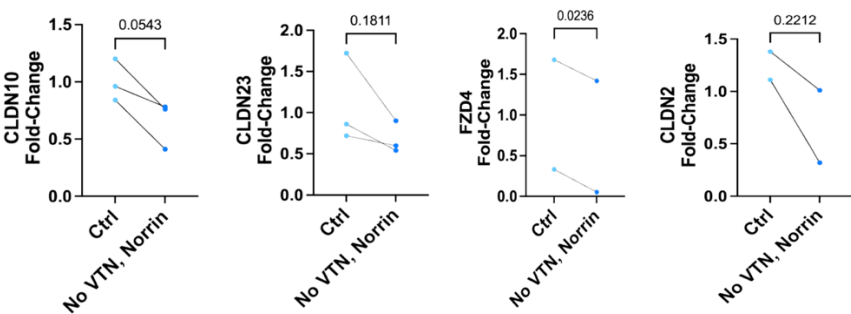
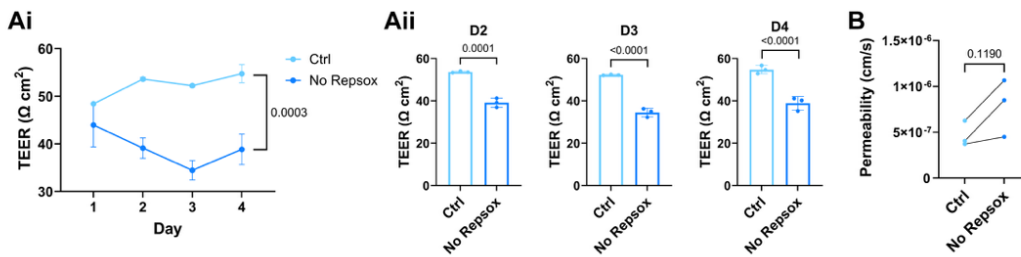
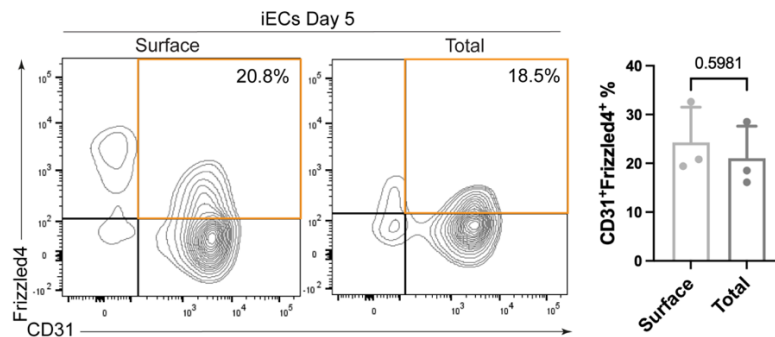


Fig. S3A-B. Barrier function measurement of iRECs treated with or without Repsox



2. The point about Frizzled-4 expression and internalization is not sufficiently clear. The authors state that ~50% of pre-iRECs undergo Fz4 internalization (Fig. 1Ciii), and that ~66% of CD31+ pre-sorted iRECs express Fz4 (Fig. 1Civ), but it is unclear whether this reflects surface or total expression. Additionally, similar surface vs. total Fz4 analysis is not provided for control iECs. In Fig. 1Cii, the addition of Norrin appears to reduce the CD31+Fz4+ surface population—possibly due to internalization—but this interpretation remains speculative. A side-by-side comparison of surface and total Fz4 in both iRECs and iECs would help clarify whether Fz4 internalization is specifically induced by Norrin signaling or reflects general heterogeneity in the differentiation process.

We thank the reviewer for bringing up this important point. The Fz4 expression shown in Fig 1Civ represents surface expression, and we have updated the figure legend and main text to clarify this. In addition, we performed flow cytometry analysis of both surface and total Fz4 expression in iECs on day 5 of the differentiation (**New Fig. S1D**). Unlike iRECs, iECs showed no significant difference between surface and total Fz4 expression, indicating that Fz4 internalization is specific to iRECs and likely reflects activation of the Norrin/Fz4 signaling pathway.



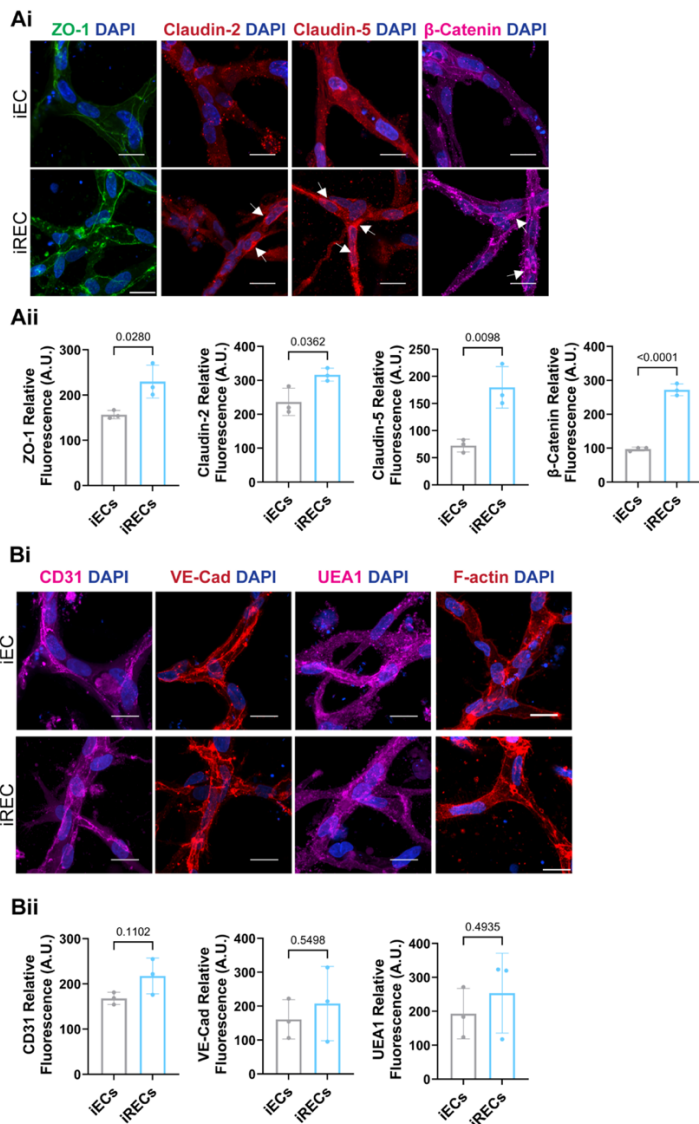
3. Overall, there is limited evidence distinguishing iRECs from control iECs. The main phenotypic differences appear to be the expression of Claudin-2 and Occludin (Fig. 1), but these observations are not supported by quantitative measurements such as western blotting, flow cytometry, or mean fluorescence intensity from immunostaining. Without such data, it is difficult to assess the robustness and extent of the reported differences. On the transcriptomic side, the authors identify several differentially expressed genes—such as FZD4, IGF2, CLDN10, CLDN23, VTN, and FN1 (Fig. 3)—but do not benchmark these against primary HRECs or retina-specific vascular datasets. As a result, it remains unclear how closely iRECs approximate native retinal endothelial cells. It would also strengthen the conclusions to examine how these gene expression patterns change when Norrin/vitronectin or RepSox are individually excluded.

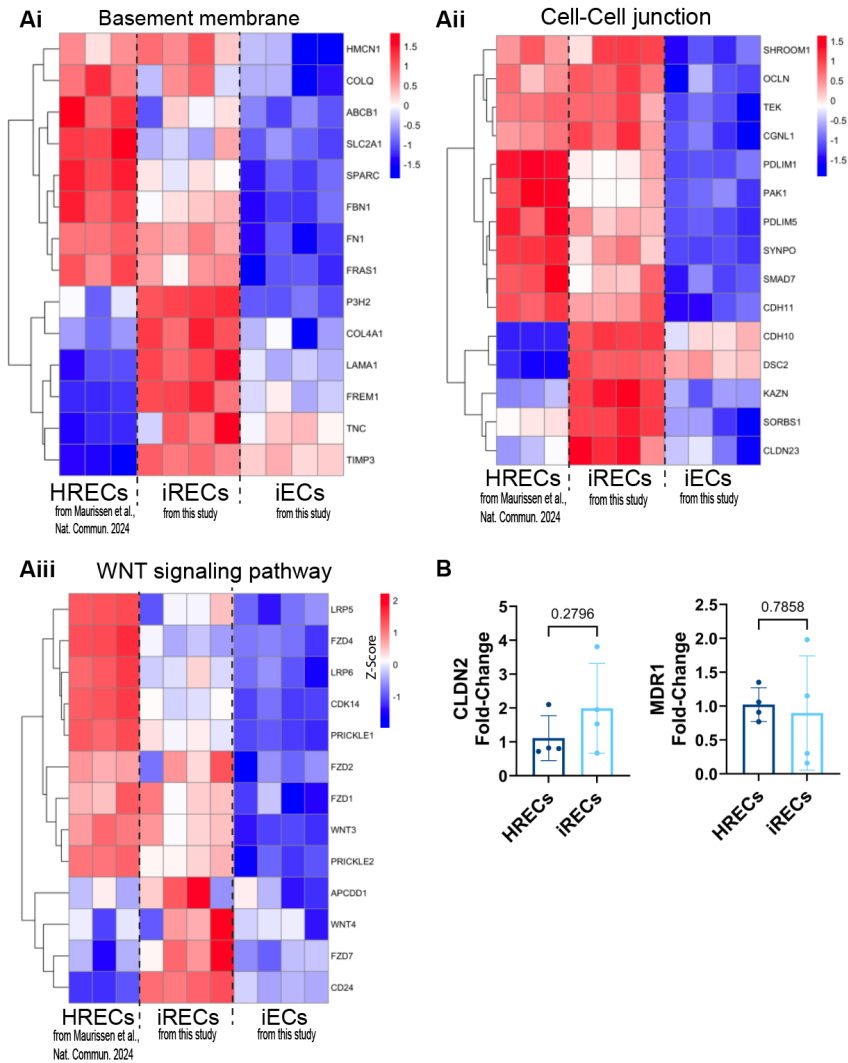
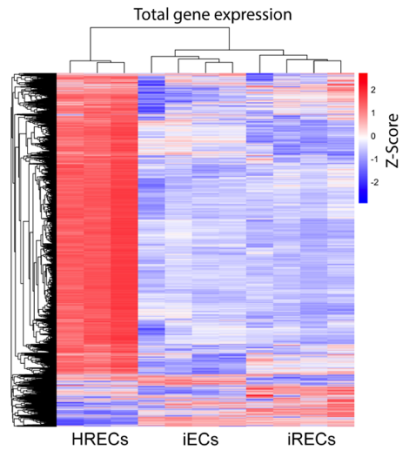
We thank the reviewer for this thoughtful and detailed comment. To strengthen our characterization of iRECs, we have now included quantitative analyses of barrier functionality and junctional and endothelial markers (New Fig. S6-9), providing functional and protein-level validation of the phenotypic differences between iRECs and iECs observed in Fig. 1. These data confirm that the enhanced expression of junctional proteins in iRECs is robust and reproducible, supporting the distinct barrier functionality and junctional phenotype conferred by retinal-specific cues.

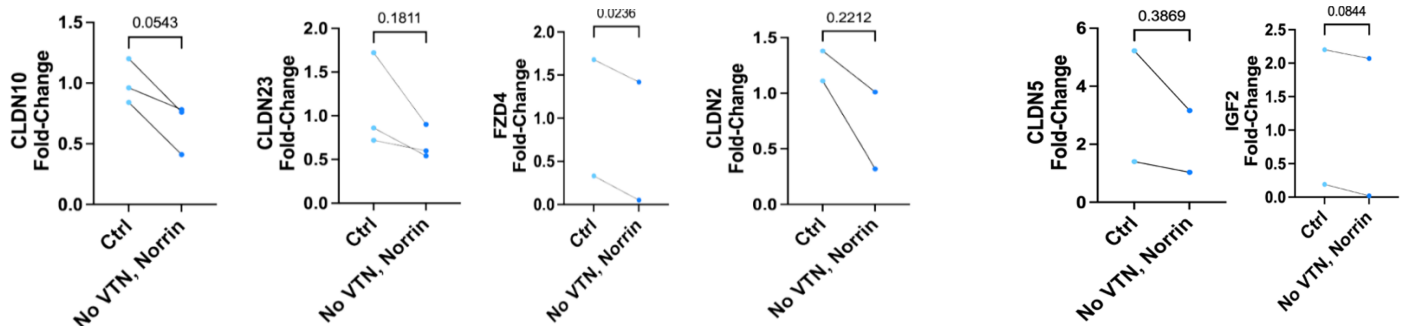
To benchmark our transcriptomic data, we compared iRECs to human primary retinal endothelial cells (HRECs) from Maurissen et al. (Nat Commun, 2024). Although global heatmap similarity was not complete, this is likely due to donor variability and culture differences, the iRECs exhibited overlapping expression across multiple retinal endothelial gene sets, underscoring their alignment with human retinal identity (New Fig. S12A). We further investigated the retinal endothelial genes CLDN2 and MDR1 by RT-qPCR and confirmed that iRECs possessed expression patterns similar to HRECs (New Fig. S12B).

Finally, to assess the contribution of factors included during differentiation, we performed RT-qPCR at day five of iREC differentiation with or without Norrin and vitronectin supplementation (New Fig. S1E). The iRECs differentiated without Norrin and vitronectin had decreased expression of the retinal EC marker, FZD4, compared to control. Their expression of the retinal EC-associated genes, CLDN10, CLDN23, CLDN2, CLDN5, and IGF2 expression was also trending toward a decrease relative to control, demonstrating that Norrin and vitronectin are critical drivers of retinal endothelial maturation.

Collectively, these results provide transcriptomic, proteomic, and functional evidence to distinguish iRECs from iECs and confirm that retina-specific signaling cues direct the emergence of tissue-relevant endothelial phenotypes.







4. There is no evaluation of DR-like responses in control iECs. The authors show that iRECs respond to high glucose and hypoxia (Fig. 2D–E), recapitulating features of diabetic retinopathy. However, without performing the same experiments in iECs, it is unclear whether these responses are specific to the iREC phenotype or simply reflect a general property of iPSC-derived endothelial cells. This comparison is essential to support the claim that iRECs provide a tissue-relevant model for retinal vascular dysfunction.

We thank the reviewer for this important comment. To directly assess whether the DR-like responses are specific to the iREC phenotype, we performed parallel experiments using iECs under identical diabetic stress conditions. iRECs exhibited a significant and progressive reduction in TEER from day 2 to day 4 compared to untreated controls (Fig. 2Diii, New Fig. S8D). Although iECs also showed a general decline in TEER under DR conditions, the changes were not statistically significant at individual time points (New Fig. S8E). Notably, the magnitude of barrier disruption was greater in iRECs than in iECs (New Fig. S8F), indicating enhanced sensitivity to DR-associated stress.

To further validate this observation, we quantified network morphology and junctional protein expression. Within 3D networks, DR-treated iRECs demonstrated marked reductions in network branch length, volume, and lumen diameter as well as ZO-1, CD31, VE-cadherin, Claudin-5, and β -catenin localization at cell–cell contacts (Fig. 2Ei–ii and New Fig. S9A), consistent with impaired barrier integrity. Initial testing with iECs in 2D demonstrates that there is no difference in ZO-1, Claudin-5, and VE-Cadherin junctional protein expression between DR and control conditions (N = 1) (New Fig. S8G, for reviewers only). Corroborating this, iEC networks treated with DR conditions possessed branch lengths, surface areas, network volumes, and lumen diameters consistent with the control groups (New Fig. S9B). Similarly, while CD31, VE-Cadherin, Claudin-5, and β -catenin protein expression are trending toward a decrease in these DR iEC networks, the reduction is not statistically significant (New Fig. S9C). Together, these findings demonstrate that iRECs exhibit better functional, structural, and molecular responses to DR stress compared to non-tissue-specific iECs, supporting their retinal identity and validating them as a tissue-relevant in vitro model for studying DR-associated vascular dysfunction.

Fig. 2Diii and Fig. S8D. TEER measurement of iRECs with or without DR treatment

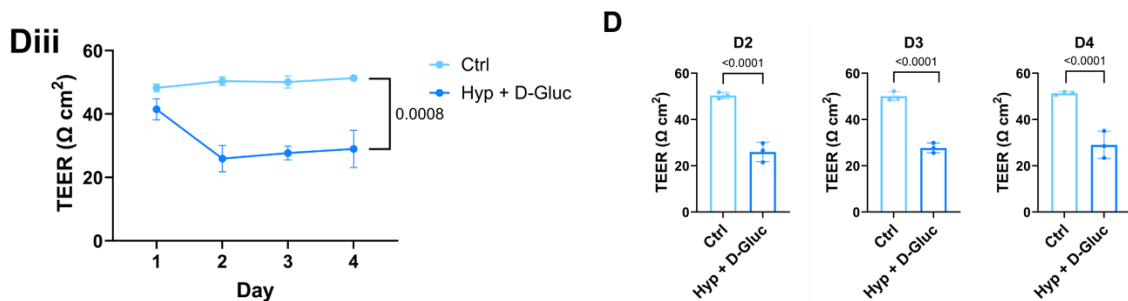


Fig. S8E. TEER measurement in iECs with or without DR treatment
Fig. S8F. Relative TEER change in iECs and iRECs after DR treatment

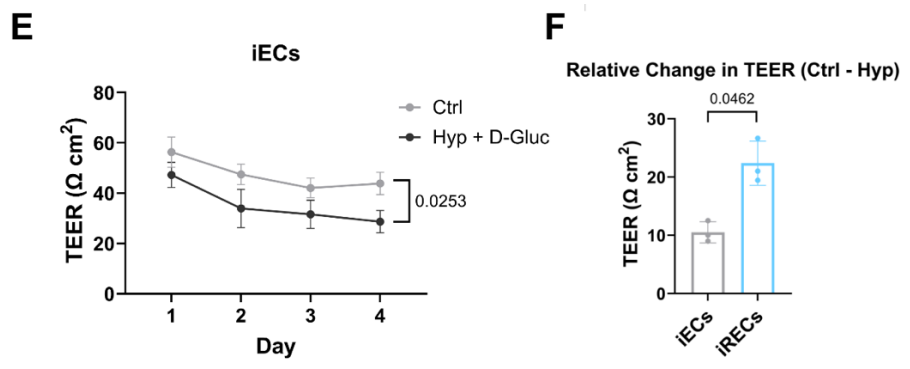


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment

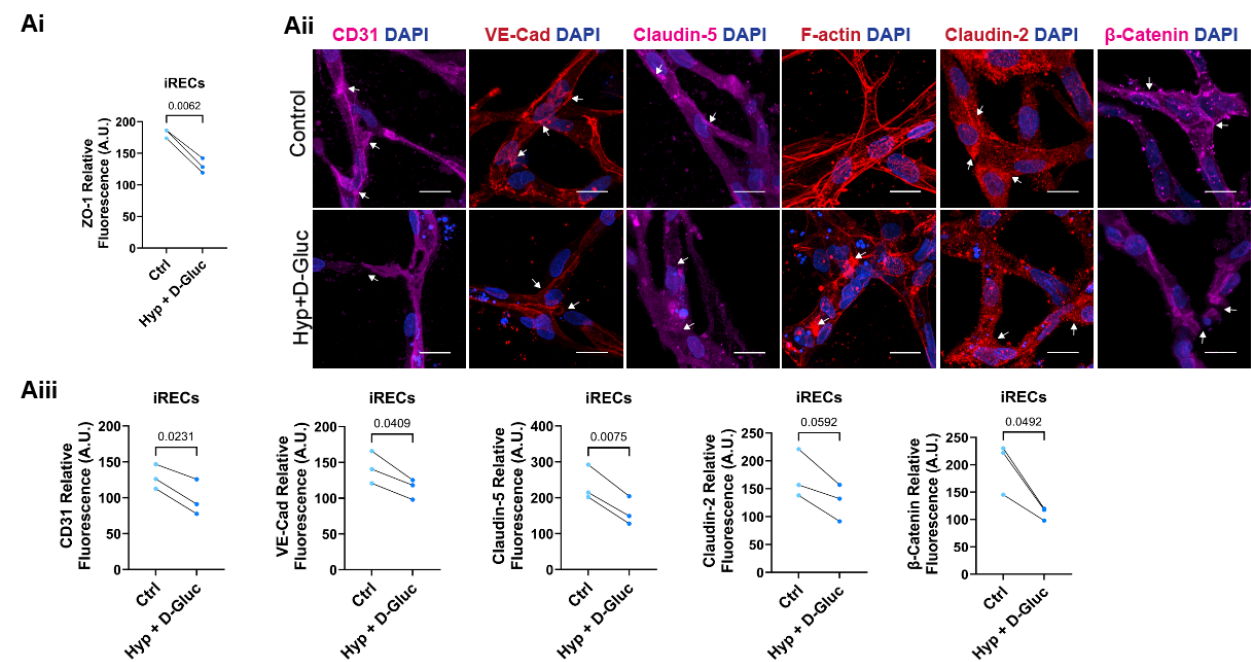


Fig.S8G. 2D iECs with or without DR treatment (Data for reviewers only)

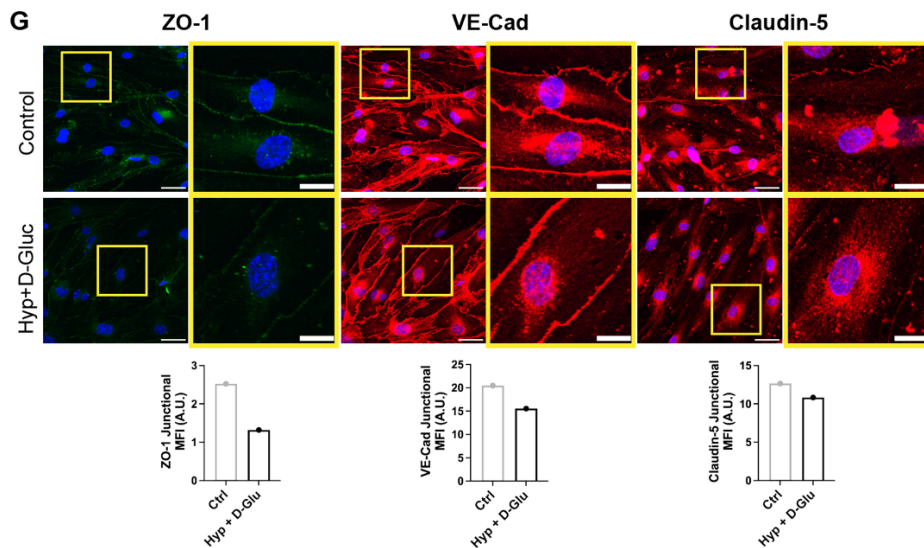


Fig.S9B. Morphology analysis of iEC vascular networks with or without DR treatment

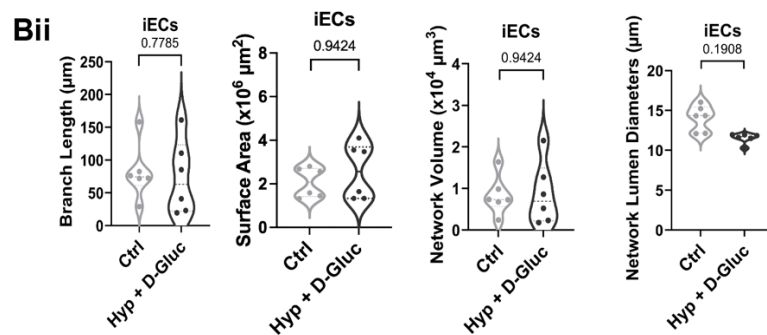
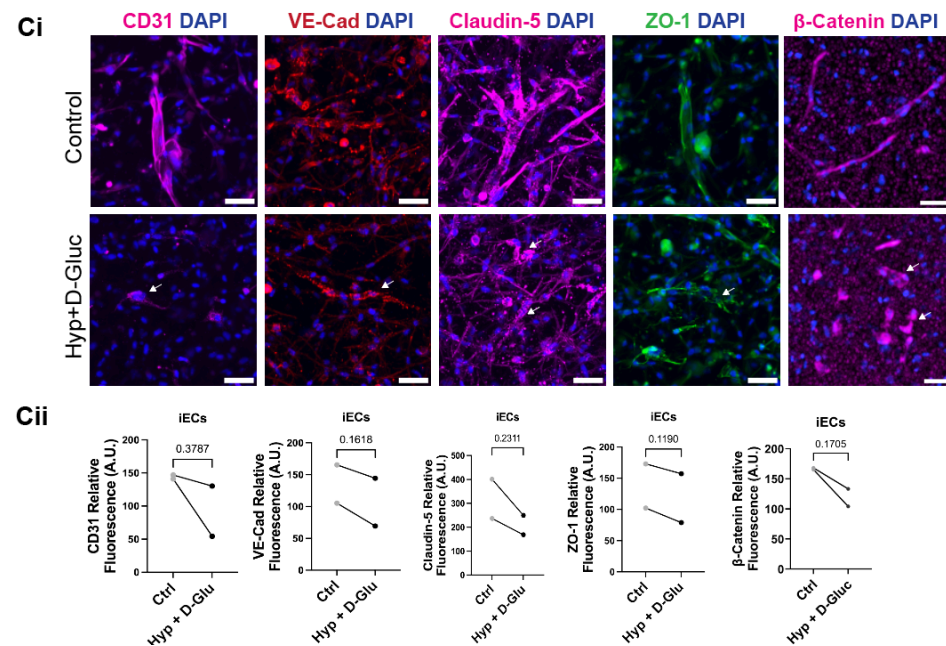


Fig.S9C. 3D iEC vascular networks with or without DR treatment

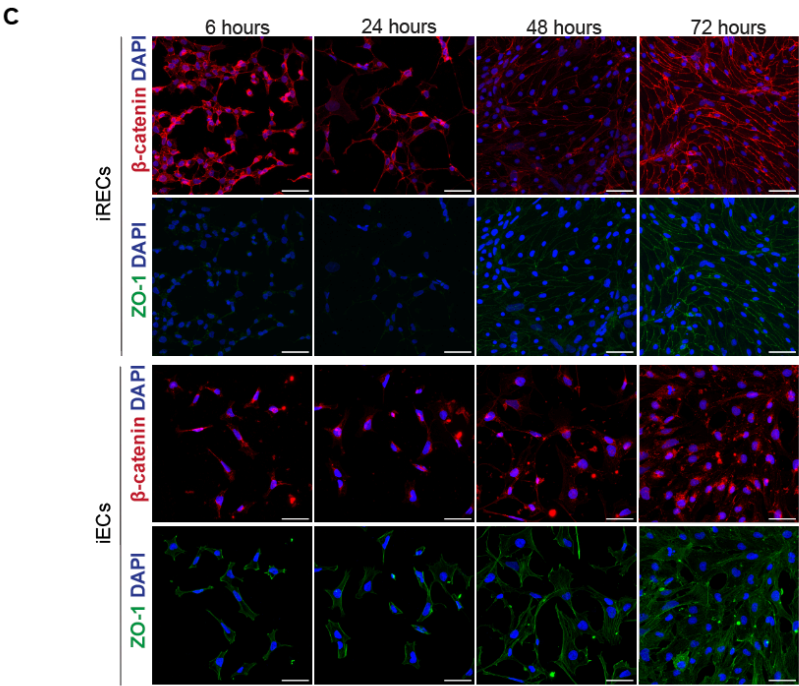


5. The transcriptomic analysis lacks tissue-specific benchmarking. While the authors show that iRECs differ from iECs and exhibit Wnt pathway enrichment (Fig. 3), they do not compare these profiles to primary human RECs or to publicly available retinal vascular datasets. As a result, the claim that iRECs “recapitulate

fundamental and developmental REC markers” feels overstated. The data suggest tissue specificity but do not confirm it. In addition, validating Wnt pathway activation at the protein level—such as through nuclear β -catenin localization—would further support the interpretation of pathway engagement.

We thank the reviewer for this thoughtful suggestion. We initially did not compare iRECs with human retinal ECs due to concerns regarding the tendency of primary cells to lose their tissue-specific phenotypic characteristic when removed from their original environment. As an alternative, we compared our samples to human primary retinal endothelial cells from Maurissen et al. (Nat Commun, 2024). The detailed results are discussed above in our response to comment 3, iRECs exhibited transcriptomic profiles similar to HRECs, including enrichment of retina-associated endothelial markers and Wnt pathway components, thereby substantiating their retinal identity.

To further support Wnt pathway activation at the protein level, we examined β -catenin localization during iREC differentiation. In pre-iRECs, β -catenin transitioned from cytoplasmic/nuclear positioning to junctional localization within 48 hours, supporting Wnt/ β -catenin signaling activation and downstream expression. This activation concided with ZO-1 expression and downregulation of ETV2, further supporting Wnt/ β -catenin pathway activation and EC maturation (New Fig. S1C). In contrast, iECs displayed minimal β -catenin activation and junctional localization. These results confirm that Norrin-induced Wnt/ β -catenin signaling is functionally engaged during iREC specification, supporting our conclusion that iRECs recapitulate key developmental and retinal endothelial features.



6. The in vivo studies omit iECs as a therapeutic control. In the OIR model, iRECs reduce vaso-obliteration and neovascularization compared to PBS-treated eyes (Fig. 4), but without a direct comparison to iECs, it is not possible to determine whether the observed benefit is specific to the iREC phenotype. Including iECs as a control would have clarified whether iRECs offer superior or tissue-specific therapeutic potential. It would also have been informative to assess in vivo phenotypic maturation of the implanted cells using human-specific markers or transcriptomic analysis, to determine whether iRECs acquire retina-specific signatures after engraftment.

We thank the reviewer for this insightful comment. To address this, we performed additional in vivo experiments including iECs as a control to directly compare their therapeutic efficacy with iRECs. Consistent with our previous findings, iEC treatment reduced both neovascularization (NV) and vaso-obliteration (VO) in OIR retinas (New Fig. S14A). Using human-specific UEA1 and CD31 staining, both iECs and iRECs were detected in close association with host vasculature and were associated with reduced vascular leakage (New Fig.4C, S14B, 4G and S14F). Moreover, rhodamine–dextran perfusion confirmed that CD31 expressing iREC-

derived lumen-like structures were functionally perfused, indicating successful vascular integration and maturation (New Fig. 4H). Notably, iRECs demonstrated better efficacy than iECs, restoring physiological vascular network with smaller segment volumes and lumen diameters while iECs did not impact these parameters (Fig. 4E and New Fig. S14D). These results emphasize that iRECs possess enhanced retinal-specific reparative potential compared to non-tissue specific iECs.

Fig. S14A-B. iECs injected OIR retina

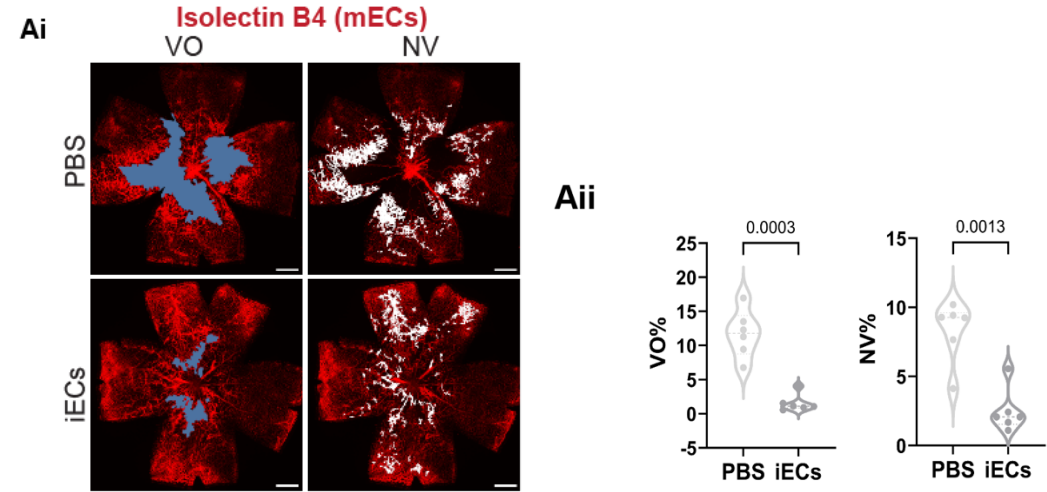


Fig 4E. Quantification of mouse vascular network segment volume and lumen diameter across PBS and iREC injected groups

Fig. 4C. iREC injected OIR retina interacting with host vasculature

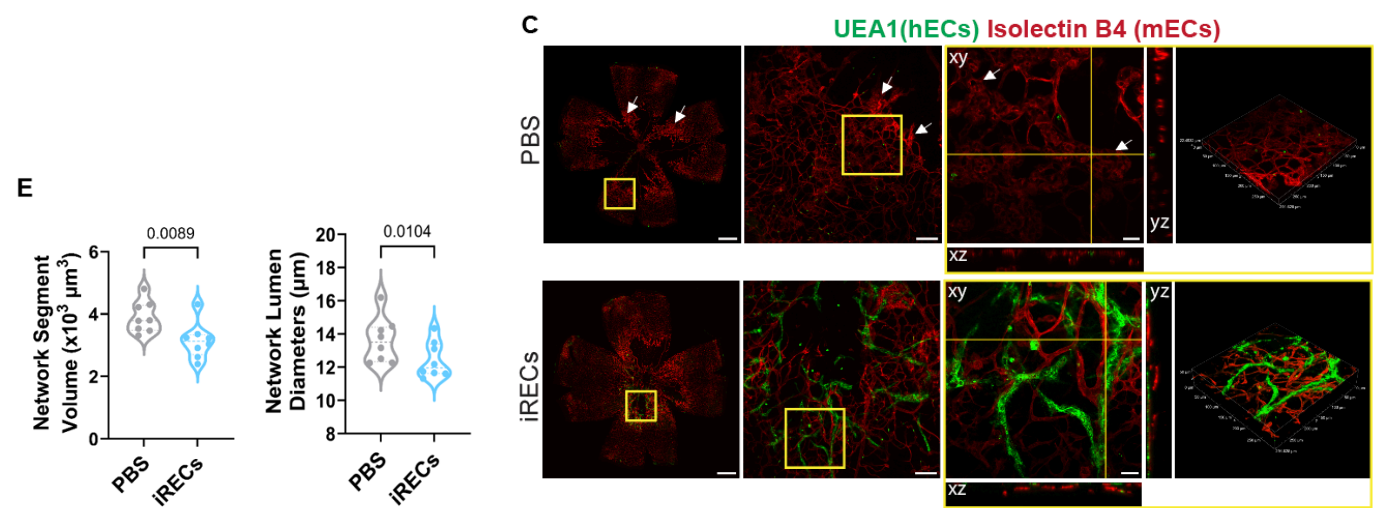


Fig. S14B. iECs injected OIR retina

Fig. S14F. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iEC injected OIR retinas

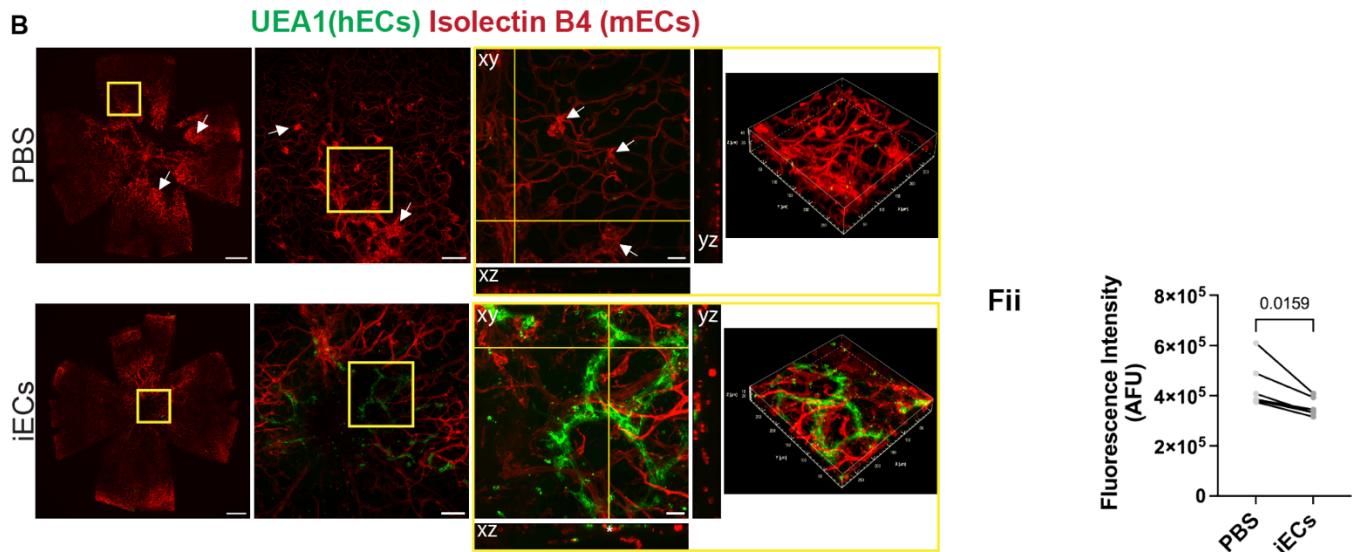
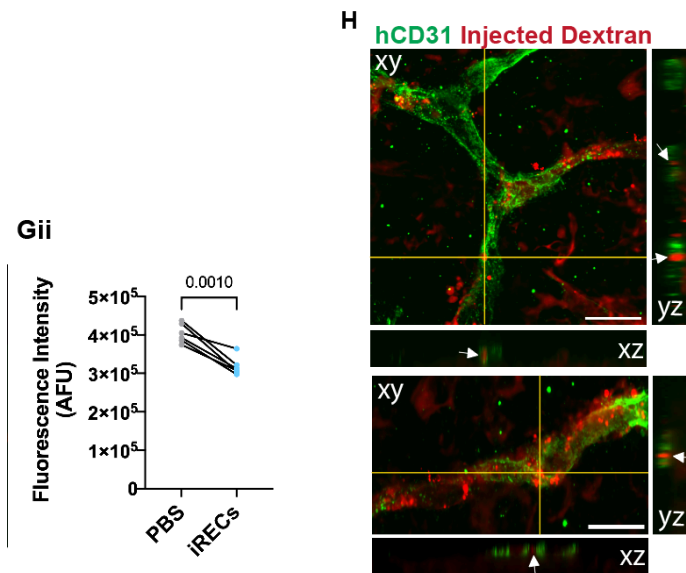


Fig.4Gii. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iREC injected OIR retinas.

Fig. 4H. iREC networks perfused by injected 70 kDa rhodamine dextran within OIR retinas



7. The microfluidic iBRB model lacks appropriate control comparisons. The authors present a perfusable iBRB-on-chip system using iRECs (Fig. 5), but without including iECs as a control, it is difficult to assess whether iRECs confer any specific functional or structural advantage. In addition, previous studies have shown that iPSC-derived ECs often require stromal or mural support to form stable vascular networks. It is unclear whether the iREC networks shown in Fig. 5A–D were formed in isolation or with supporting cells—this should be clarified.

We thank the reviewer for this valuable comment. The vascular networks shown in Fig. 5A–D were formed using iRECs alone to directly evaluate their intrinsic capacity to form perfusable vasculature relative to iECs. Although iREC and iEC network size and morphology were comparable, iRECs exhibited lower permeability and thus increased barrier properties relative to iECs, (New Fig. 5E, S15B and S15Ciii). This demonstrates that the iRECs can generate a functional and physiologically accurate iBRB, displaying their

biological fidelity and advantage over non-tissue specific ECs for iBRB modeling. When iRPCs were included during network formation, iRECs developed smaller, capillary-scale networks with further decreased permeability (New Fig. 5I-J), closely resembling human retinal microvasculature¹⁻⁵. These results highlight the retina-specific functional advantage of iRECs and the supportive role of iRPCs in establishing physiologically relevant iBRB architecture and barrier properties.

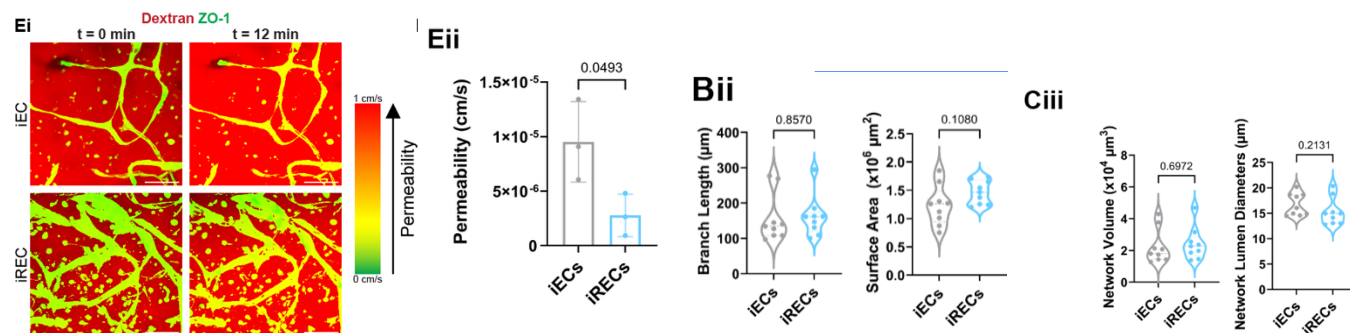


Fig. 5I. iRECs-iRPCs co-cultured MPS

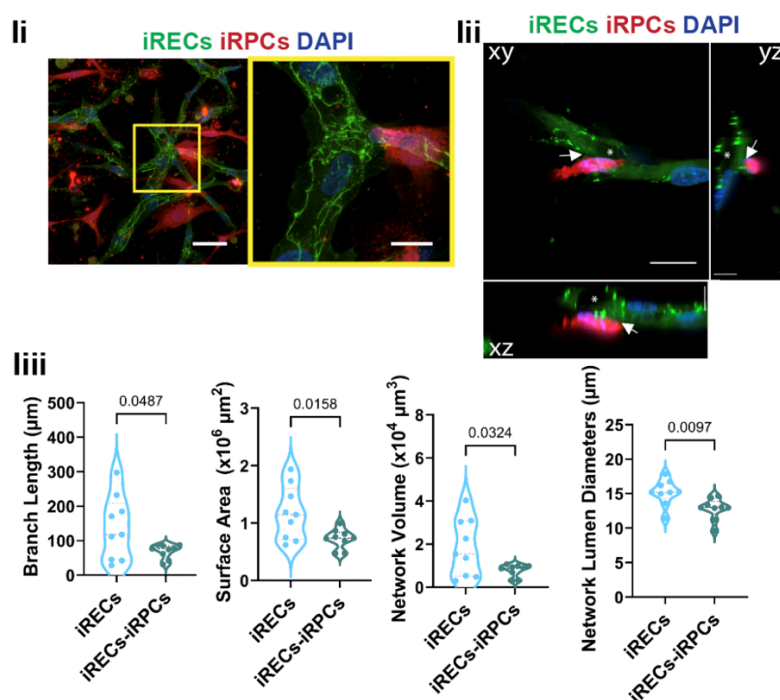
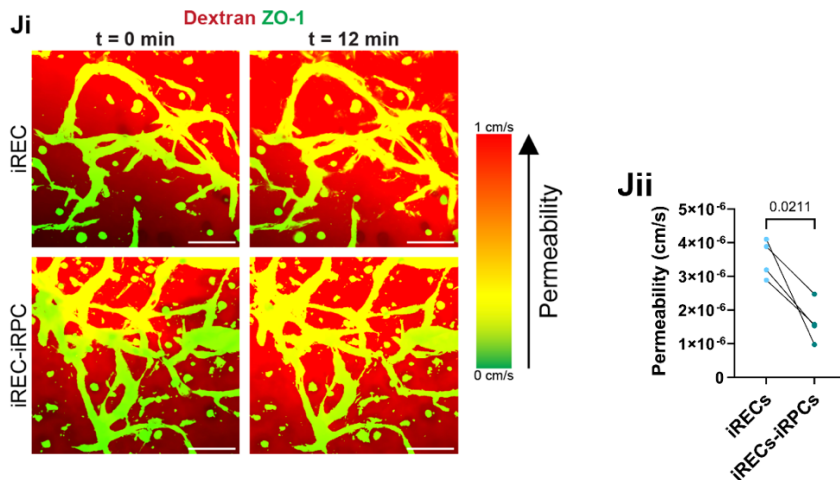


Fig. 5J. Permeability assay of iRECs-iRPCs co-cultured MPS



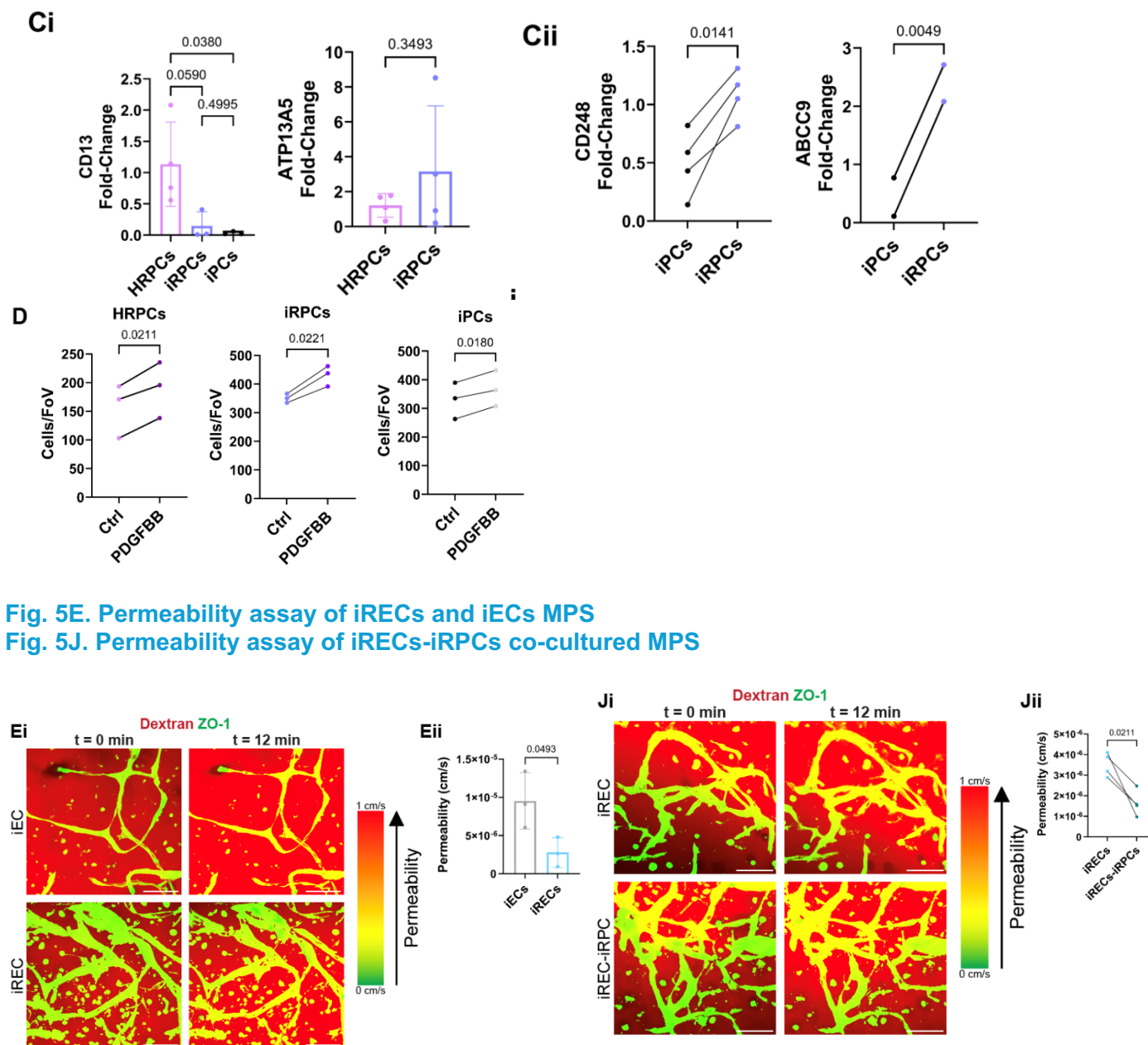
8. The characterization of iPSC-derived retinal pericytes (iRPCs) is underdeveloped. Their retinal specificity is primarily inferred from Fz4/PDGFR β co-expression (Fig. 5F–G), but this conclusion appears premature. There is no comparison to control iPSC-derived pericytes or other mesenchymal populations, and no functional assays are provided to assess pericyte competence. Moreover, the protocol for iRPC generation is introduced with minimal validation, and the role of Norrin in their specification is not explored in depth. Also, the conclusion that the iREC–iRPC co-culture “recapitulates tissue-specific retinal-like vascular networks” is not adequately supported. While the structural organization is evident, the study does not show that this model performs better than alternative combinations, such as iECs with non-retinal pericytes. The authors could reasonably state that the system recapitulates physiological endothelial–pericyte interactions, but the tissue-specific aspect of the model is not convincingly demonstrated.

We thank the reviewer for this thoughtful comment. The characterization of retinal pericytes indeed remains limited in the literature. To address this, we utilized RT–qPCR analyses to assess the retinal specificity of iRPCs at the transcriptomic level. The iRPCs expressed comparable levels of the pan-pericyte marker CD13 to HRPCs and iPCs (New Fig. S16Ci), and similar levels of the retinal-specific marker ATP13A5 to HRPCs (New Fig. S16Ci). Additionally, they exhibited elevated expression of the pericyte-associated genes CD248 and ABCC9 relative to iPCs (New Fig. S16Cii).

To investigate functionality, we performed a Transwell migration assay in the presence and absence of platelet-derived growth factor BB (PDGFBB), an EC secreted factor critical to iBRB development and thus a stimulus the iRPCs must respond to^{8–11}. The HRPCs, iRPCs, and iPCs all exhibited an increased migration to the basolateral side of the Transwell in the PDGFBB group compared to the control (New Fig. S16D), confirming that iRPCs retain key pericyte function.

Morphological and functional analyses in the MPS revealed that iREC–iRPC co-culture interactions contributed to a decrease in vascular network branch length, surface area, volume, and lumen diameter relative to the iREC only group (New Fig. 5liii and S16F), recapitulating iBRB network morphology and the luminal size of human iBRB capillaries^{1–5}. The iRPCs also increased the barrier properties of the iREC networks, decreasing their 70 kDa rhodamine dextran permeability to a level consistent with in vivo permeability¹² (New Fig. 5J). Taken together, this demonstrates the retinal-specific signature and biological fidelity of the iRPCs and their ability to develop morphologically and functionally relevant iBRB networks.

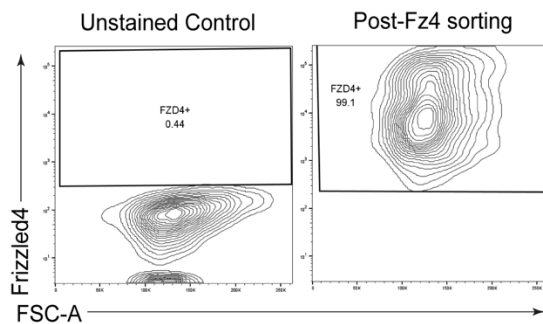
We acknowledge that additional characterization would further strengthen the study and have noted this as a limitation in the revised “*Limitations of the study*” section.



Minor Concerns

In Fig. 1, it would be helpful to report the efficiency of the Fz4+ sorting step. Providing post-sort purity (e.g., via flow cytometry) would help assess the robustness of the protocol and the homogeneity of the iREC population.

We thank the reviewer for bringing up this important point. To address this, we performed flow cytometry using labeling check reagent APC (Miltrenyi Biotech, #130-122-228) to verify the purity of the cells after the Fz4 sorting step. We found that 99.1% of the cells were Fz4-positive post-sort, confirming the robustness of the protocol and the homogeneity of the iREC population. (Data for reviewers only)



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Response to reviewers

General comments:

To increase the rigor and confirm the reproducibility and accuracy of our work, we have updated our RT-qPCR data for Fig. S1E, network analysis for Fig. S9Bii, and fluorescence intensity data for Fig. S8D and S9Cii to include additional biological replicates. We added a ZO-1 stain to Fig. S2D to clearly delineate cell-cell borders to confirm Dil-LDL uptake. We also provided more accurate representative images for Fig. S9Bi and Ci and changed the color scheme in Fig. S16 Eiii-F to ensure consistency with Fig. 5li-ii and S16Ei-ii.

Reviewer comments:

Reviewer #1:

The authors have conducted key additional experiments and provided new data supporting the superior performance of iRECs (compared to generic iECs) in in vitro disease modeling. Several concerns raised in the previous review have been addressed. However, although the in vitro benefits of iRECs are now well demonstrated, their claimed superiority in vivo remains unsubstantiated. A number of important issues persist:

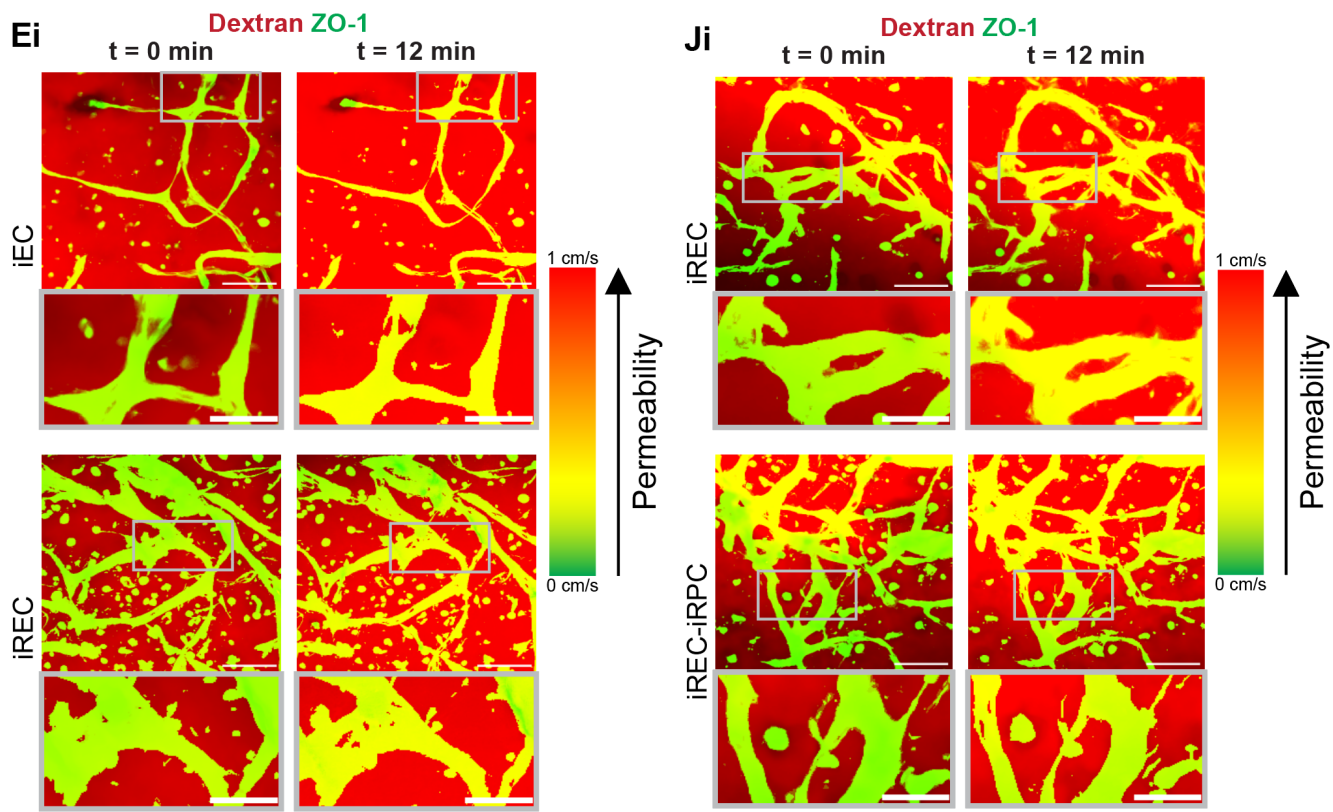
1. Vessel permeability assessment in the MPS

The method used to assess vessel permeability is unclear and requires further clarification. The authors state that they filled the hydrogel with fluorescent tracer and quantified fluorescence intensity changes within the vascular network, which, if I interpreted correctly, reflects reverse permeability. If this is indeed the intention, representative images showing differences within the vasculature should be provided, as the current images illustrate only the interstitium. Even if images reveal differences, the relevance of this assay to physiological permeability remains questionable and should be justified.

We thank the reviewer for this important question. Your interpretation is correct, we flooded the collagen-I hydrogel with 70 kDa rhodamine dextran, enabling us to quantify the permeability into the networks. To do this, we followed a standard and exhaustively validated protocol that quantifies the change in network fluorescence to establish permeability¹. As a modification to this protocol, we calculated the fluorescence intensity difference within the networks over time rather than the matrix as the flooding of the hydrogel with dextran prior to imaging was used to investigate the permeability into the networks rather than out of the networks. As the protocol has been validated for quantifying fluorescence intensity for permeability regardless of the direction of dextran flow, the use of the equation for permeability into the network is accurate and thus physiologically relevant. Previous studies have also demonstrated that the permeability into and out of cells and networks can be calculated and the permeability in either direction is indistinguishable²⁻⁵, further supporting the physiological relevance of our permeability assays and calculations.

Within the representative maximum intensity projection immunofluorescence images from Fig. 5Ei and Ji, ZO-1-GFP denotes iEC or iREC networks. As a result, the change in green network color across timepoints $t = 0$ min and $t = 12$ min, where the green networks become more yellow/red, is a result of the rhodamine dextran accumulation within the networks and thus represents network permeability into the networks rather than just displaying interstitium. We have updated the figure legend to clarify this. We also updated Fig. 5Ei and Ji to include inset images to enable an easier visualization of the network color change between $t = 0$ min and $t = 12$ min.

We added this clarification to the figure legend.



2. Discrepancies between Fig. S15 Ei/Eiii and Fig. 5Ei/Ji

Figures S15 Ei and Eiii look markedly different from Fig. 5Ei and Ji. The authors should explain this discrepancy. Additionally, why was the perfusion method, which is standard in the field not used to assess permeability directly?

Fig. 5Ei and Ji are representative real-time images that were taken during the permeability assay at $t = 0$ min and $t = 12$ min without fixing and washing. Prior to imaging, we delivered $20 \mu\text{L}$ of transport buffer to one media channel and $70 \mu\text{L}$ of TB buffer supplemented with $100 \mu\text{g/mL}$ 70 kDa rhodamine dextran to the other media channel of a device. This generated an interstitial flow that enabled the rhodamine dextran to diffuse throughout and fill the collagen type-I hydrogel. Once dextran was homogeneously distributed across the hydrogel, we began imaging the samples with the first image stack representing t_0 ($t = 0$ min). Image stacks at the same location as t_0 were taken 12 minutes later to complete the permeability assay. As rhodamine dextran was present throughout the hydrogel for both image timepoints, the images possessed rhodamine dextran signal surrounding and within ZO-1 networks.

In contrast, to visualize vascular perfusion in Fig. S15E, devices were washed with PBS for 5 minutes on a rocker to remove excess dextran within the hydrogel. Devices were then fixed in 4% formaldehyde for 20 minutes followed by 3 PBS washes. These wash steps removed the perivascular rhodamine dextran within the hydrogel without washing out the dextran located in the networks, resulting in excessive perivascular rhodamine dextran being absent from the images in the Fig. S15E. This generates the distinct images from Fig. S15E without perivascular rhodamine dextran relative to Fig. 5Ei and Ji. As perfusion imaging was performed after fixing and washing the cells, permeability measurements could not be calculated.

To ensure the difference between the images and the assays are clear, we updated the permeability and vascular perfusion descriptions within the *iBRB-on-a-chip permeability assay* section in the paper.

The permeability assay we used was designed to address the challenge associated with using collagen-I hydrogels. We encapsulate iRECs and iECs within collagen-I hydrogels to generate the MPS as collagen-I is a naturally occurring ECM component within the retina. This represents a critical advantage of our system and its ability to recapitulate the physiological environment compared to the commonly used fibrin-based hydrogels,

which lack the native retinal ECM component⁶⁻¹⁴. The “standard” perfusion method the reviewer is referring to is used for 3D models made with fibrin hydrogels. However, collagen-I and fibrin hydrogels are composed of different materials and thus they possess different mechanical and structural properties, with collagen-I hydrogels possessing greater susceptibility to contractility. While we initially tried to perfuse the networks with 70 kDa rhodamine dextran using the luminal perfusion method, we were unable to perfuse them without using bulk hydrogel perfusion due to the mechanical and contractile properties of collagen-I hydrogels. As a result, we could not accurately calculate fluorescence change outside of the networks and thus network permeability using the luminal perfusion approach. To address this, we use the well-established approach to determine permeability¹ and flood the hydrogels with 70 kDa rhodamine dextran to quantify dextran permeability from the surrounding hydrogel into the networks.

3. Gene expression comparisons between HRECs, iECs, and iRECs

The gene expression analysis shows that iECs and iRECs are far more similar to each other than either is to HRECs. This is concerning, as many genes exhibit an inverse expression pattern relative to HRECs. The authors should address this more thoroughly, discussing implications for the retinal identity and fidelity of iRECs.

We thank the reviewer for raising this important point regarding the transcriptional similarity between iECs and iRECs relative HRECs and its implications for retinal EC identity. RNA-Seq data for iECs and iRECs were generated in parallel by the same vendor using identical library preparation and sequencing pipelines. In contrast, the HREC RNA-Seq dataset was obtained from a published study¹³. Although DESeq2 normalization and VST were used to reduce technical variance within datasets, direct cross-study comparisons remain susceptible to batch effects and bias associated with the sequencing system utilized, which contribute to the apparent difference in global gene expression patterns. To highlight this limitation, we stated that “An additional challenge comes with comparing published primary HREC and iPSC-derived brain EC RNA-Seq datasets with our datasets as there is a potential for batch effects and bias associated with the sequencing system utilized” within the manuscript’s *Limitations of the study* section.

Despite these limitations, iRECs exhibit selective transcriptional similarity with HRECs in functionally relevant pathways critical to retinal-EC identity, including basement membrane, cell-cell junction, and Wnt signaling related genes. On the contrary, this similarity is not observed to the same extent in iECs. To this end, while iRECs do not recapitulate the complete transcriptional profile of HRECs, they express genes fundamental to the retinal signature of HRECs to a greater degree than iECs, supporting their retinal identity and genomic fidelity. Although iECs and iRECs share broad transcriptional features likely due to their common iPSC-derived origin, the retinal-specific expression patterns present in iRECs are limited or absent in iECs, corroborating the physiological relevance and accuracy of iRECs.

To clarify these points and strengthen the interpretation, we revised the *Discussion* section to emphasize the pathway-specific evidence underlying iREC identity.

4. Claims regarding “enhanced retina-specific reparative potential of iRECs”

The data do not sufficiently support this conclusion.

- In Fig. S14, the iEC networks (panel Fi) appear far more robust than either PBS or iREC conditions (panel E). Based on visual assessment and quantitative parameters, there are very few differences between iECs and iRECs in reparative potential.
- The two experimental batches differ substantially.
 - o Batch 1 (original submission): PBS vs iREC, with VO = 20% in PBS controls.
 - o Batch 2 (revision): PBS vs iEC, with VO = 10% in PBS controls.

This suggests a substantially milder insult in Batch 2, which may mask differences between PBS and iECs. Importantly, variability in PBS controls is 2× between batches, whereas variability in treated groups (iEC/iREC) is lower, indicating high model variability overall.

- Only two parameters showed significant differences for iRECs: network segment volume (noting that iECs were nearly significant, $p = 0.059$ —was power calculated?) and vessel diameter, whose relevance to regeneration is unclear.

- VO, NV, and Dextran fluorescence intensity differed significantly for both iECs and iRECs, while branch length and surface area did not differ for either group. This pattern suggests more similarities than differences between iECs and iRECs in vivo.

Taken together, these findings may reflect assay variability rather than a true enhanced reparative potential of iRECs in comparison to iECs. While the authors were overall cautious in their claims, stating mostly that iRECs have a regenerative ability, the authors are encouraged to either provide additional evidence through a direct head-to-head comparison of iECs and iRECs within the same experimental batch, or remove claims of in vivo superiority (page 34, last sentence) and include this as a limitation of the study.

We thank the reviewer for these important and helpful points. While the OIR mouse model is the standard and most widely used model for studying DR angiogenesis/neovascularization and the discovery and testing of therapeutics^{15,16}, there is inevitable variability within and across batches. This is due to differences in pup-to-pup handling, weight, nursing, light exposure, etc., which can not be completely accounted for and mitigated. While a direct comparison between iECs and iRECs within the same mouse would provide the best mechanism to evaluate their therapeutic and reparative capabilities, we are unable to perform this direct iEC and iREC comparison as there is a need for a PBS control in the contralateral eye to confirm initial injury. A bilateral transfer of biological material across eyes has also been documented¹⁷, further preventing a reliable direct comparison of iRECs and iECs within the same mouse due to the potential of cell migration and paracrine signaling confounding results and hindering data analysis and statistical comparisons. We have removed the claim that iRECs have enhanced capabilities relative to iECs and have updated the *Limitations of the study* section.

5. Concerns with perfusion shown in Fig. 4H

The perfusion data are not convincing. There appears to be as much (or more) red signal outside the vessels as inside. The small region where red overlaps with green seems localized and potentially artifactual. If the vessels are leaky—making perfusion difficult to assess—this should be explicitly addressed. The authors should clarify:

- Why such extensive extravascular red signal is present,
- Whether the chosen time point is appropriate, and
- How leakage affects interpretation of perfusion success.

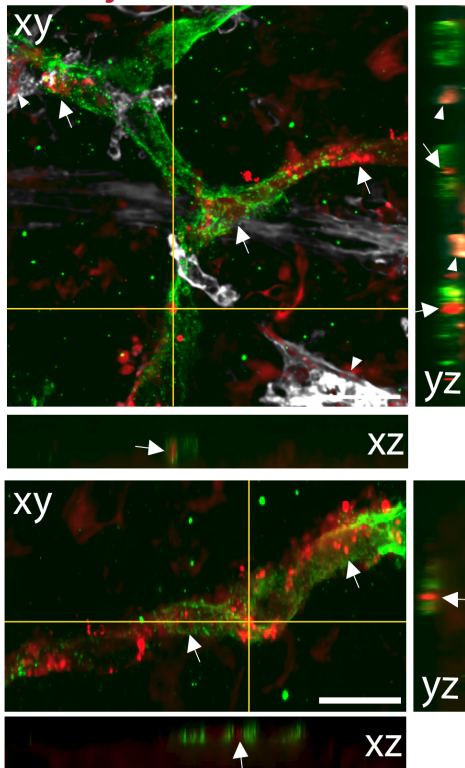
We thank the reviewer for this insightful question. Fig. 4H shows a representative retinal region containing iREC-derived vascular networks after iREC injection. This experiment was designed to determine 1) if iREC networks are functionally connected to the host vasculature and 2) whether iREC treatment reduces retinal vascular permeability compared with PBS controls. The presence of rhodamine dextran within the lumina of iREC networks demonstrates that these structures are perfusable and connected to the host vasculature. The extravascular rhodamine dextran signal is associated with the native mouse vasculature and iREC-derived vessels and is expected under the experimental conditions. In the original figure, we focused on highlighting iREC network perfusion and therefore did not include the marker for host vasculature. To address this, we updated the figure to include the Isolectin B4 stain for mouse ECs. Although there is some residual background rhodamine dextran signal present, this is expected since we performed the permeability assay on P17, when vaso-obliteration and neovascularization are at their peak in the model. This timepoint was chosen as it is the standard in the field for therapeutic evaluation, including permeability assays¹⁸⁻²¹. This timepoint also corresponds to the most extreme case of retinal injury, allowing us to evaluate the full reparative capacity of the iRECs. Accordingly, increased vascular permeability is anticipated, and a fully sealed EC barrier is not expected in either host or iREC-derived vessels at this time point.

Retinal vascular permeability was quantified by extracting extravascular 70 kDa rhodamine dextran present within the entire retina using N,N-Dimethylformamide and measuring fluorescence intensity. As retinal vascular permeability quantification is calculated based on the amount of extravascular rhodamine dextran within a retina, the leakage within each retina is fundamental to establishing its permeability. The less leakage present, the lower the permeability calculated, and thus the greater the vascular repair following iREC treatment. For perfusion of iREC networks, we considered any iREC network with rhodamine dextran encapsulated within its lumina perfusable.

We have revised the text and Fig. 4 to address and clarify the perfusion and permeability assay and outcomes.

H

CD31 (hECs) Isolectin B4 (mECs)
Injected Dextran



General Comments

- Many graphs use line plots to compare discrete conditions (e.g., PBS-iPECs, PBS-iECs, Ctrl-PDGFBB, iPCs-iRPCs). This is inappropriate, as line plots imply continuous or paired data (e.g., before-after). This raises concern about incorrect statistical treatment, particularly since figure legends do not specify the statistical tests used. If the conditions were indeed paired, that should be explicitly clarified—but based on the experimental design, this seems unlikely.

We thank the reviewer for raising this concern. As clarification, all line plots or before-after plots correspond to data that we performed two-tailed paired Student t-tests on as they all represent paired data. All assays where cells from the same biological replicate were exposed to different conditions in parallel underwent paired testing. The *in vivo* assays were also analyzed utilizing two-tailed paired Student t-tests as each individual mouse was injected with PBS in one eye and iRECs or iECs in the contralateral eye. Paired testing was utilized to account for batch effect and sample bias.

We included an explanation of which data underwent paired analyses within the *Statistics* section of the paper where we stated “Two-tailed paired Student t-tests were performed to statistically compare all GLUT1 uptake and diabetic retinopathy assay data. All before-after plots resemble paired data and denote two-tailed paired Student t-test analyses”. To clarify this section and avoid confusion, we have updated this description to include line plots and *in vivo* assays.

- All statistical methods and sample sizes (n) should be included in each figure legend to allow proper evaluation of the results.

We thank the reviewer for highlighting this important point. The statistical methods and sample sizes utilized within each figure are included within the *Statistics* section of the manuscript (see below). If the sample size for a given figure/result is not N = 3, n = 3, this is specified within the figure legend.

Statistics:

Biological replicates (indicated as N) are defined as separate differentiation replicates and technical replicates (indicated as n) come from the same N cultured in different tissue culture wells. All *in vitro* quantifications are N = 3 with n = 3 unless otherwise indicated in the figure legends. For *in vivo* experiments, mice number in each experiment is indicated as n. All bar graphs represent means $\pm$ SD. Graphpad Prism (v10.2.3.) and JMP 17 were utilized for statistical analysis. Two-tailed paired Student t-tests were performed to statistically compare all GLUT1 uptake, diabetic retinopathy, and *in vivo* assay data. All before-after plots, also known as line plots, resemble paired data and denote two-tailed paired Student t-test analyses. The p-gp analyses were performed utilizing two-tailed one sample Student t-tests. For TEER assays, we utilized two-factor repeated measures ANOVA to compare cell types. Post-hoc Tukey's tests were then performed to analyze individual days. Two-tailed unpaired Student t-tests were performed as appropriate for all other statistical comparisons. Significance levels were set at $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $****p \leq 0.0001$.

Reviewer #2:

The authors have followed our suggestions.

We thank the reviewer for taking the time to review all of our edits and updates and for their helpful suggestions and feedback during the review process.

Reviewer #3:

The authors have responded thoroughly to the prior critiques, and the manuscript is clearly improved. The added controls, functional comparisons, and clarification of claims strengthen the overall rigor. The conclusions are now more measured, and the key outcomes are supported by the data. I am satisfied with the revisions. One small point that could be acknowledged in the Discussion is that, at a global transcriptomic level, iRECs remain closer to iECs than to primary HRECs, which is expected at this stage. This does not detract from the significance of the work but would help to frame the findings realistically.

We thank the reviewer for thoroughly reviewing all of our edits and updates and for their beneficial suggestions and feedback during the review process. To clarify the transcriptomic analysis and properly frame the findings, we have updated the *Discussion* section to acknowledge that while iRECs do not completely reproduce the entire transcriptional profile of HRECs, they demonstrate targeted alignment in key retinal pathways, including those related to the basement membrane, cell-cell junctions, and Wnt signaling genes. This indicates that iRECs possess a physiologically relevant retinal signature. Although iRECs and iECs share many transcriptional characteristics owing to their shared iPSC-derived origin, iECs lack the extent of retinal-specific gene expression alignment with HRECs that iRECs exhibit. These findings support the retinal identity and genomic integrity of iRECs.

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Reviewer comments:

Reviewer #1:

Degree of advance

The field of endothelial organotypic identity and plasticity has recently attracted considerable attention. Seminal questions related to EC organotypic identity in engineered tissues and regenerative medicine are unanswered. Key questions relate to whether ECs can adopt the organ-specific characteristics of their implantation site or if organ-specific ECs are required for tissue/organ function in transplantation. Papers addressing these seminal questions should draw significant attention and be highly cited. While Lin et al tangentially address some aspects of this exciting area of research, the lack of appropriate controls (generic ECs) for functional readouts in both in vivo and in vitro models have dampened the enthusiasm for the findings.

There is certainly a methodological advance: the development of an EC differentiation protocol to generate retina-like ECs. However, not enough evidence was given, in its present form, to provide significant fundamental, mechanistic, or performance-wise advances – which would increase significantly the impact of this paper.

Implication of the findings

As stated by the authors, 'there is an increasing need to develop tissue-specific ECs for more precise disease modeling and effective tissue regeneration and repair'. However, there was no demonstration in this manuscript that the retina-like ECs more precisely model the retina EC behavior or show tissue-specific function in vitro and in vivo when compared to generic ECs.

While generating retina-like ECs and studying their general characteristics certainly has merit, the greatest advancement would be to provide evidence that it is advantageous to use tissue-specific ECs (in this case iRECs) in vitro (as even native primary tissue-specific cells lose phenotype in culture) and in vivo compared to 'generic' ECs or cells from other tissues. Given that the prevailing dogma is that ECs are plastic and can adapt to the local environment, the significant higher cost and time needed to differentiate tissue-specific ECs needs to be justified. However, authors failed to provide convincing evidence of the advantages of using iREC, as most assays performed lacked a direct comparison to generic ECs or ECs of different origins. Without this, the advancement to the field is modest and future adoption by others will be limited. Towards that goal, authors should provide appropriate controls for functional assays.

Major criticism

1- Lack of 'generic' EC controls in both in vitro and cell transplantation assays limits the conclusions

We thank the reviewer for highlighting this important point. To strengthen our study, we included iECs as controls in several key assays to further demonstrate the biological fidelity of the iRECs and their ability to model DR and repair the ischemic retina. iECs were incorporated as a control in:

- GLUT1 and P-gp transporter assays (New Fig. S6C-D),
- Permeability and TEER assay (New Fig. S6B),
- All DR modeling experiments (New Fig. S8E-G, and S9B),
- The OIR mouse model (New Fig. S14), and
- The MPS study (New Fig. S16C-E).

These experiments have enabled us to compare iRECs to iECs to better highlight the unique retinal-specific properties of iRECs and their overall robustness.

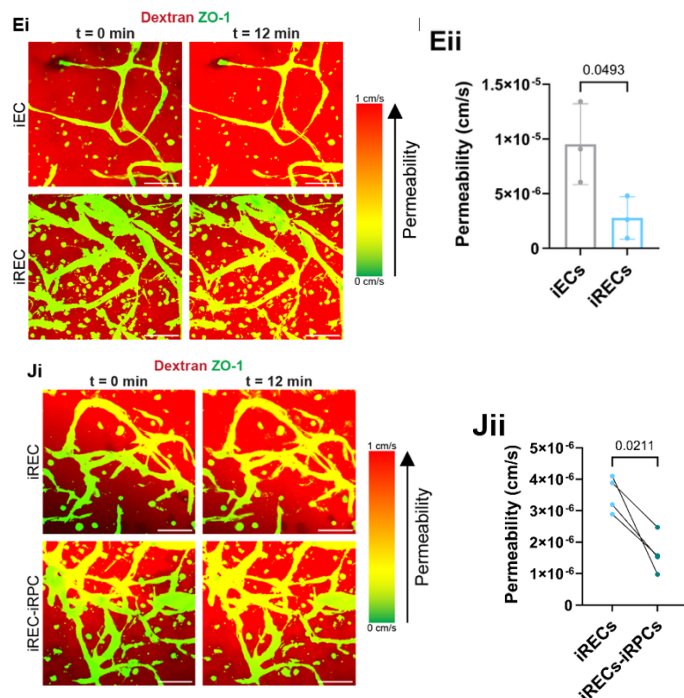
These comparisons consistently demonstrate that iRECs possess retinal-specific properties and functionalities. *In vitro*, they exhibit robust GLUT1 and p-gp transporter activity, enhanced barrier function with higher TEER and lower permeability, and greater sensitivity to DR-associated stress with pronounced reductions in barrier properties and junctional protein expression. *In vivo*, iRECs more effectively restore vascular network morphology in the OIR model. In the MPS, iREC networks possess increased junctional protein expression and display lower permeability than iEC networks, reflecting physiologically accurate barrier function.

Collectively, these results confirm that iRECs outperform generic iECs in modeling retinal-specific vascular phenotypes and DR, reinforcing the biological fidelity and utility of the iREC platform.

2- It is unclear why the authors elected to test the vessel formation in MPS and not provide assessment of barrier function under fluid flow – which, as they point out themselves “enabling the study of tissue function” and this is the best application of MPS that is also relevant for their hypothesis. iRECs permeability coefficient should be provided and compared to iECs in the chip, under flow, to assess if they indeed enable improved barrier function. While TEER is a good method, it lacks functional assessment, which can be achieved in the chip (see Pham Lab on a chip 2017, Chen lam Chip 2023 etc.).

We thank the reviewer for this important comment. We included a permeability assay to assess the barrier function and establish the permeability coefficient of iECs, iRECs, and iREC-iRPCs within the MPS. To quantify permeability, our method uses a slightly modified approach based on an established study from Hajal et al., Nat. Protoc., 2022. Briefly, 20 μ L of transport buffer was delivered to one media channel and 70 μ L of transport buffer containing 100 μ g/mL 70 kDa rhodamine dextran to the opposite channel. This generated interstitial flow, enabling rhodamine dextran to perfuse and fill the collagen type-I hydrogel. Once the hydrogel was homogeneously filled, imaging began (t_0), and the same location was imaged 12 minutes later to complete the assay. Fluorescence intensity changes within the vascular networks were quantified over time to determine permeability, rather than measuring the surrounding hydrogel.

Using this assay, iREC networks were successfully perfused by 70 kDa rhodamine dextran and exhibited significantly lower permeability compared to iEC networks (New Fig. 5E). Co-culture with iRPCs further enhanced barrier properties, reducing 70 kDa rhodamine dextran permeability to levels consistent with *in vivo* measurements (New Fig. 5J). Collectively, these findings demonstrate that incorporation of iRPCs into the iBRB MPS strengthens EC barrier function and support the formation of physiological relevant vascular networks.



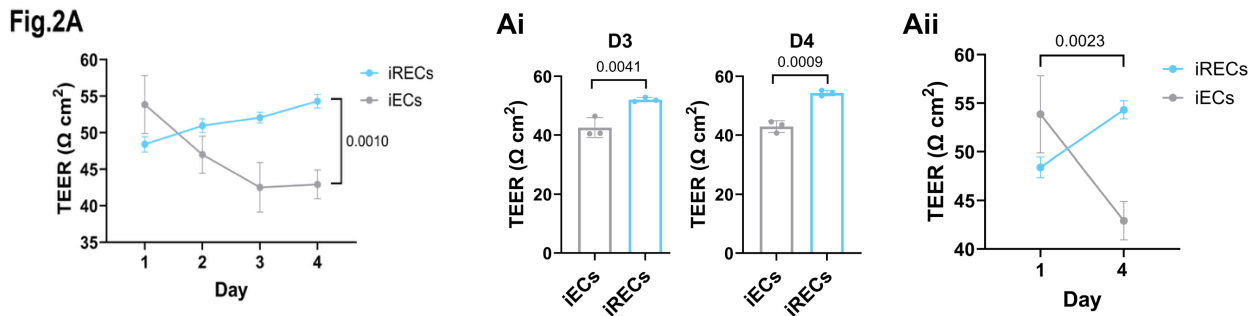
3- On TEER: what was the media used for the experiments? Methods describe that iECs were cultured in media with 50ng/ml of VEGF while iRECs in the same media but without VEGF – see excerpt below. Given that VEGF is well known for inducing EC permeability, differences in TEER could be due to the different culture media used. Results must be provided for assays performed with the same media for all cells.

“The purified CD31+ iECs were plated on a type-I collagen-coated plate and expanded and maintained in ECGM supplemented with 10 μ M SB-431542, 50 ng/ml VEGF-A, and 10 μ M Y-27632. After 24 hours, the media was changed to remove Y-27632. After that, media was changed every other day.”

“iRECs were plated on a type-I collagen-coated plate and expanded and maintained in ECGM supplemented with 10 μ M RepSox, 10% platelet poor human serum (Millipore Sigma), and 10 μ M Y-27632. After 24 hours, the media was changed to remove Y-27632 and then the media was changed every other day.”

We appreciate the reviewer’s comment. We reperformed the TEER and permeability assay with both iRECs and iECs cultured in iREC media. The methods section has been updated to include these new details.

With the new conditions, we observed higher TEER in the iRECs relative to the iECs across the four days (**Fig. 2A**), displaying enhanced barrier properties. We specifically identified that the iRECs possessed increased TEER values on day 3 and day 4 (**New Fig. S6Ai-ii**), suggesting a physiologically relevant maturation of iREC barrier integrity over time absent from iECs. This validates that the iRECs contain high barrier functionality consistent with the iBRB, supporting their biological fidelity and utility.

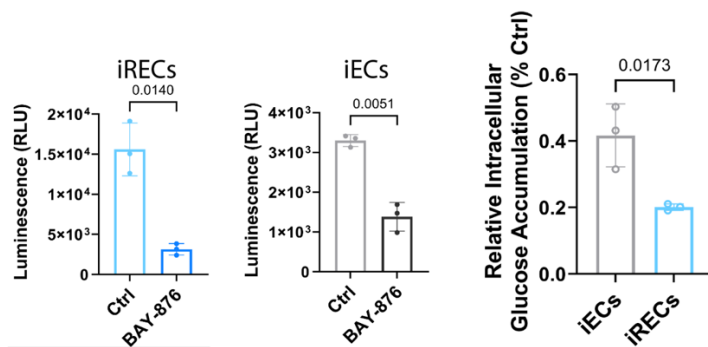


4- There are not enough details in the methods to reproduce the findings, so methods need to be improved substantially. Cell seeding density at thawing and passaging and for assays need to be provided, how many cells were collected in 100ul of FACs buffer, etc.

We thank the reviewer for this comment. We have updated the methods section to include more detailed information, including cell numbers used during stem cell culture, differentiation and the various assays.

5- It would be good to have a comparison between iREC and iECs for glut1 inhibition to show specificity

We thank the reviewer for this comment. We added new Fig. S6C to include iEC in the GLUT1 transporter assay. The GLUT1 receptor plays a crucial role in the retina. The GLUT1 transporter is the main facilitator of glucose transport, which is critical to meeting the energy demand in the retina. When treated with glucose, iRECs exhibit a 5-fold higher baseline glucose uptake level (1.5×10^4 vs. 3×10^3) than iECs (Fig. 2B and New Fig. S6Ci). When the iRECs were treated with the highly selective GLUT1 inhibitor, Bay-876, glucose uptake in iRECs was reduced by 80% while iEC uptake was lowered by only 60% (Fig. S6Cii). The greater GLUT1 activity and transporter functionality within iRECs supports their retinal specificity.



6- Fig 2 Cii, Eiii; S3a, b, Fig 5C are not really useful as it is difficult to draw conclusions from 1 image. For rigour, assessments must be provided as in Fig 2Dii.

We thank the reviewer for this comment. We agree that quantification is essential for rigor. We have now added quantification for Fig. 2Cii, 2Eiii, S3A, S3B (New Fig. S7A-B), and 5C (New Fig. S15D).

These analyses reveal that iREC networks exhibit elevated membrane-associated ZO-1, Claudin-2, Claudin-5, and β -catenin at cell-cell contacts relative to iEC networks, consistent with their iBRB-specific phenotype and enhanced barrier properties (TEER, permeability, and stability; New Fig. S7A). In contrast, canonical EC markers—CD31, VE-Cadherin, UEA1—show comparable expression between iRECs and iECs, confirming endothelial identity (New Fig. S7B).

Under diabetic retinopathy (DR) conditions, 3D iREC vascular networks showed decreased junctional expression of ZO-1, CD31, VE-Cadherin, Claudin-5, and β -catenin, indicating compromised barrier function, whereas Claudin-2 remained unchanged, consistent with prior in vivo observations (New Fig. S9A).

Furthermore, in MPS, iRECs consistently displayed higher junctional ZO-1, Claudin-2, and β -catenin than iECs (New Fig. S15D), reinforcing their retinal-specific barrier integrity and phenotypic accuracy.

Fig. S7. 3D iEC and iREC vascular networks

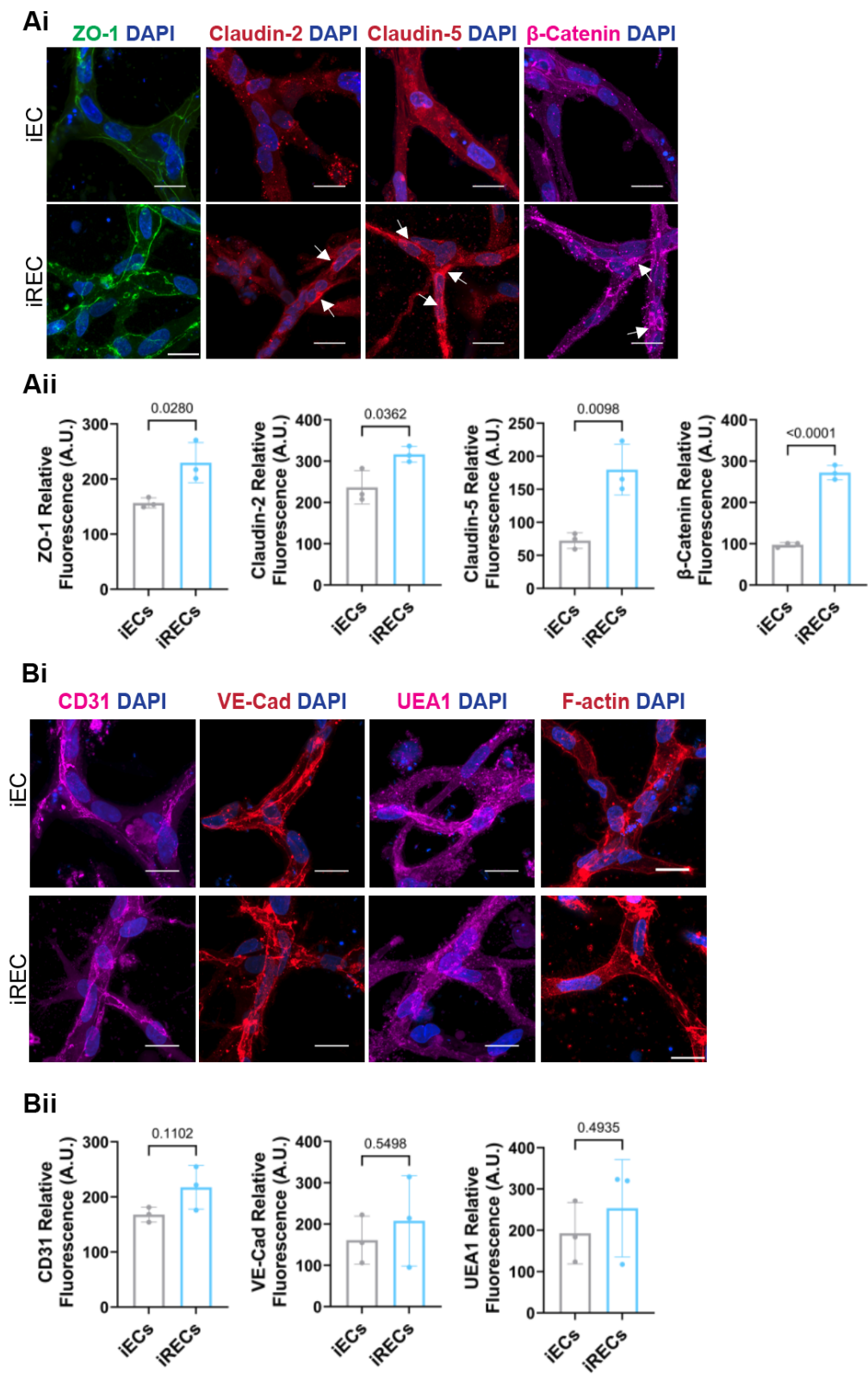


Fig. S9. 3D iREC vascular networks with or without diabetic treatment

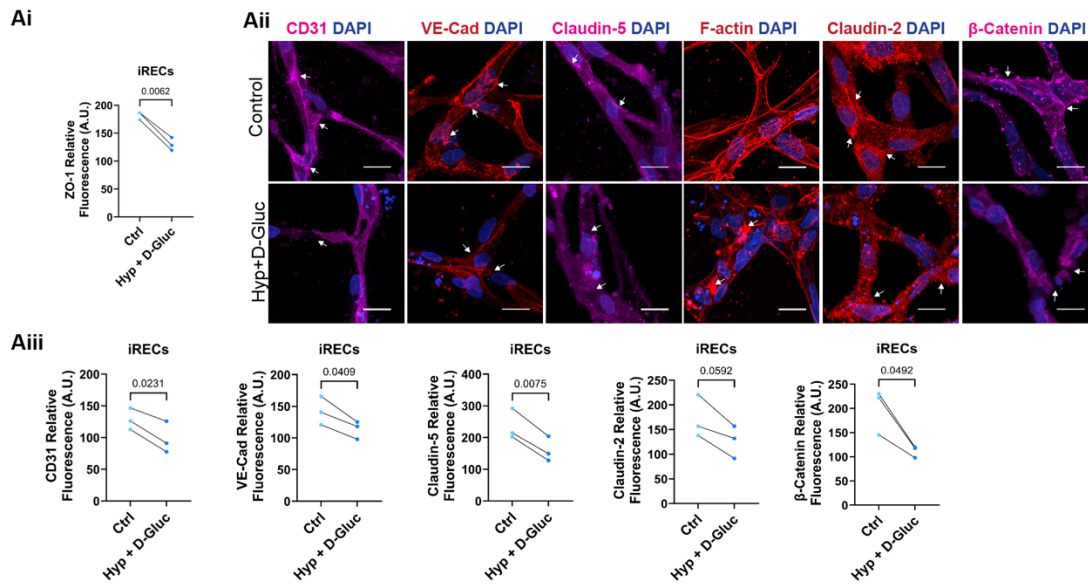
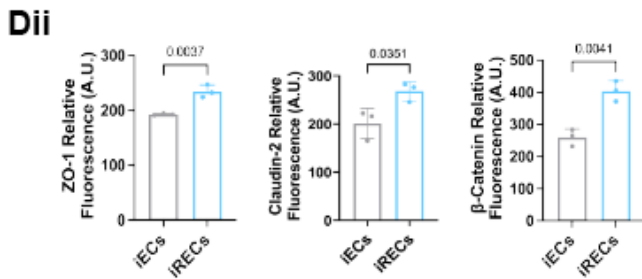


Fig. S15. Quantification of junctional protein expression in iEC and iREC vascular networks within MPS



7- Diabetic retinopathy conditions used in the manuscript are extreme: 30mM of glucose is extremely high and then there's the presence of 1% O₂ – for up to 72h. I am amazed any cells were still alive. Assessment of cell viability (e.g. live dead stain) would be useful to understand the extent of the DR treatment. Authors should also provide a better justification for using such an extreme model. Moreover, comparison with iECs should be done. Are iRECs better at modeling diabetic retinopathy? In which aspects? This would be extremely valuable for the field and highlight the advantages of using tissue-specific ECs.

We thank the reviewer for this thoughtful comment. The glucose concentration used to recapitulate DR was based on method previously utilized within the literature for *in vitro* DR modeling (Maurissen et al., Nat Commun, 2024; Eyre et al., Exp Eye Res, 2020; Kaur et al., Am J Physiol Cell Physiol, 2023; Chandrakumar et al., Diabetes, 2024). A glucose concentration of 30 mM resembles grade 4 serum hyperglycemia, a life-threatening condition that necessitates immediate treatment. Modeling this stage of hyperglycemia with our iRECs and systems can help accelerate therapeutic development to improve treatment efficacy.

To address the concern that this extreme condition could lead to decrease cell viability, we assessed cell viability in 2D using flow cytometry. We observed comparable survival between iRECs and iECs under DR conditions (New Fig. S8A), confirming that the treatment is not cytotoxic. Functional and structural analysis in both 2D and 3D cultures revealed that iRECs are more sensitive to DR-associated stress than iECs (New Fig. S9). iRECs showed significantly reduced TEER, decreased junctional protein expression (ZO-1, CD31, VE-Cadherin, Claudin-5, β-catenin), and decreased network morphology (Fig. 2D-E, New Fig. S9A, and New Fig. S8D). In contrast, iECs did not contain significant differences in TEER at individual time points and had reduced changes in TEER compared to the iRECs (New Figs. S8E-F). Initial testing with iECs in 2D demonstrate that there is not a difference in ZO-1, Claudin-5, and VE-Cadherin junctional protein expression between DR and control conditions (N = 1, New Fig. S8G, not in the manuscript) Corroborating this, iEC networks treated with DR conditions possessed branch lengths, surface areas, network volumes, and lumen

diameters consistent with the control groups (New Fig. S9B). Similarly, while CD31, VE-Cadherin, Claudin-5, and β -catenin protein expression are trending toward a decrease in these DR iEC networks, the reduction is not statistically significant (New Fig. S9C).

These results indicate that iRECs faithfully recapitulate structural and functional features of DR, whereas generic iECs show limited responsiveness, highlighting the retinal specificity and modeling advantage of iRECs.

Fig. S8A. Cell viability analysis of control and diabetic-treated iECs and iRECs.

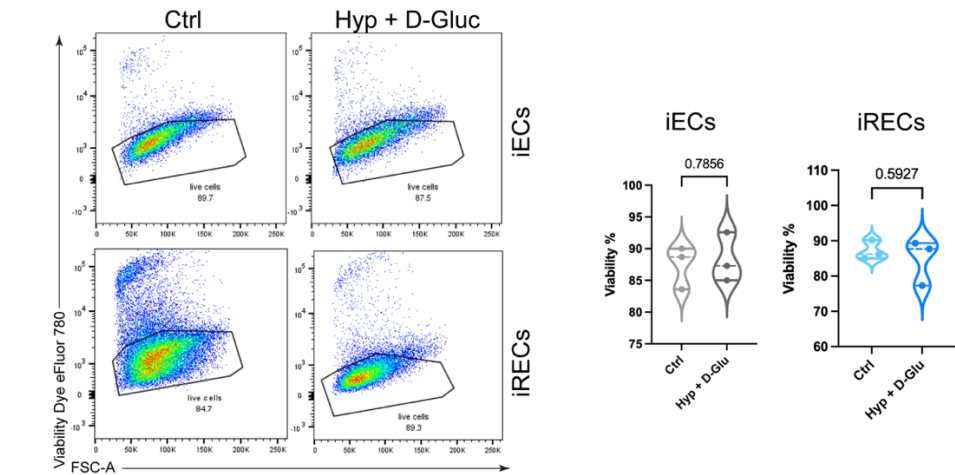


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment

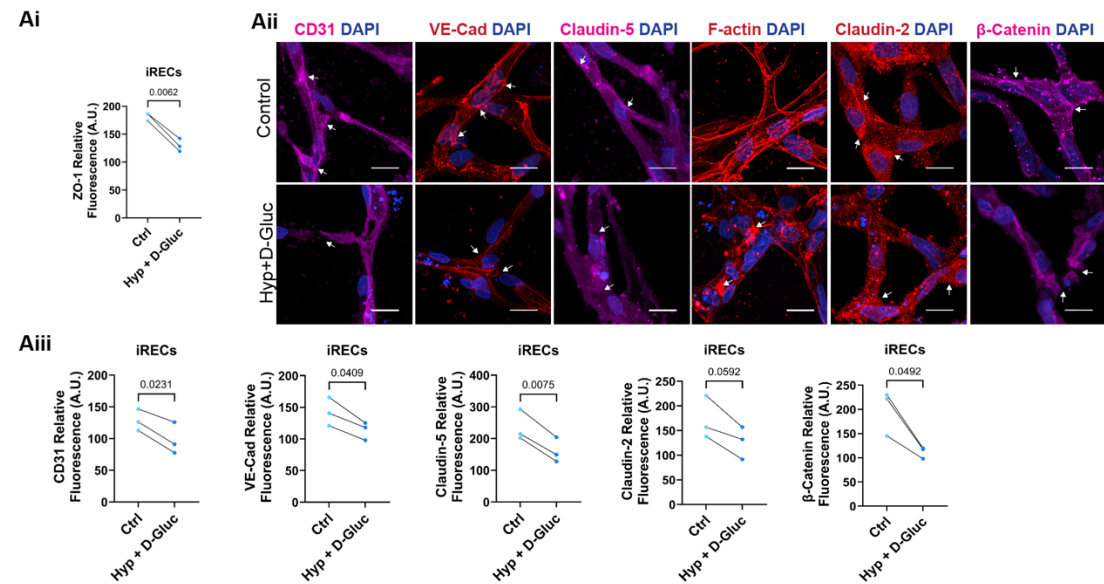


Fig. 2Diii and Fig. S8D. TEER measurement of iRECs with or without DR treatment

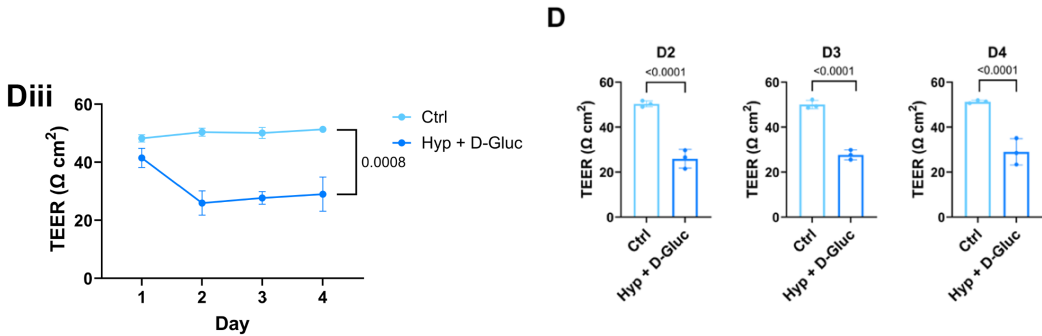


Fig. S8E-F. TEER measurement of iECs with or without DR treatment. And relative TEER change in iECs and iRECs after DR treatment.

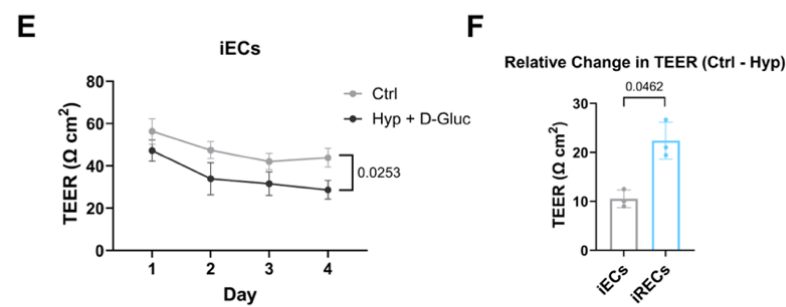


Fig.S8G. 2D iECs with or without DR treatment (Data not shown in the manuscript)

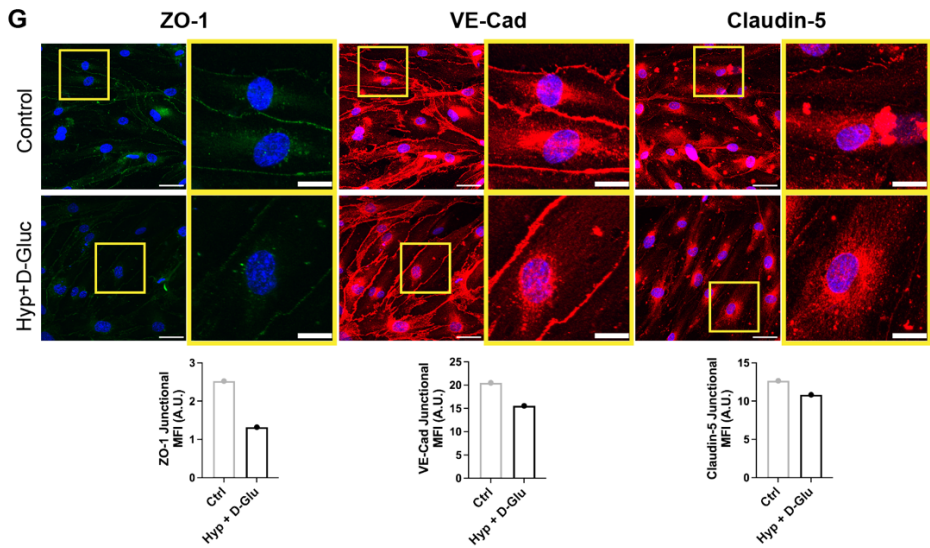


Fig.S9B. Morphology analysis or iEC vascular networks with or without DR treatment

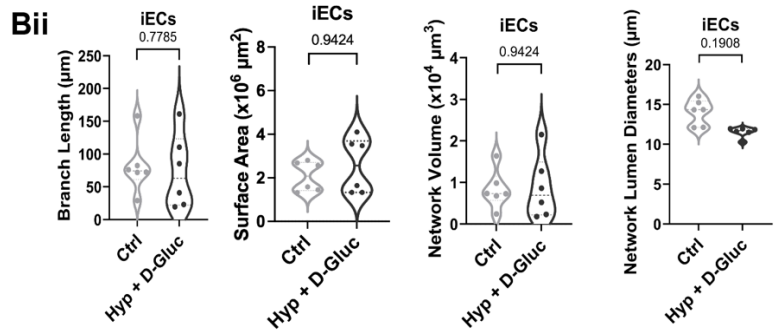
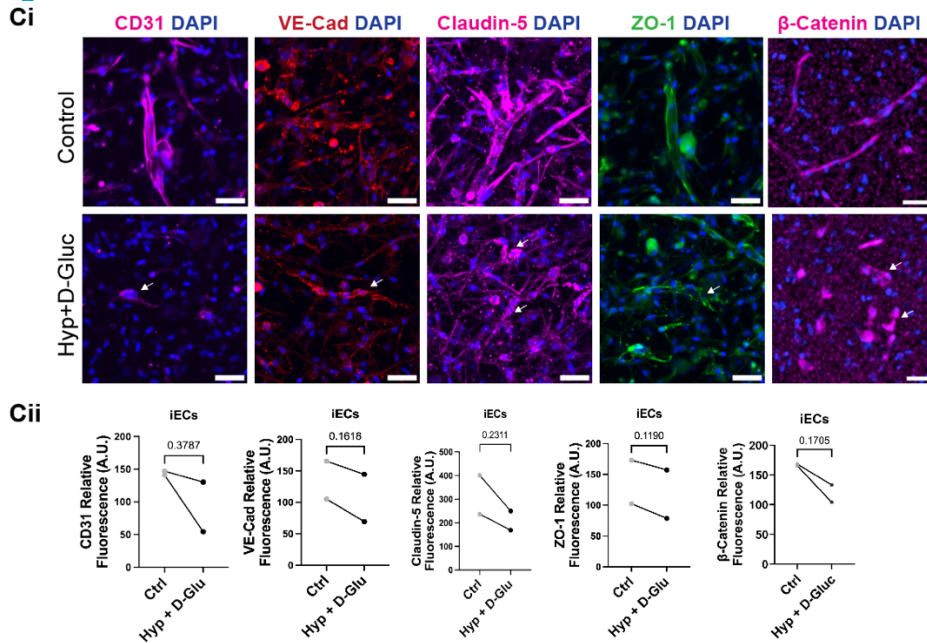


Fig. S9C. 3D iEC vascular networks with or without diabetic treatment



8- Was the hypothesis that DR would induce loss of Claudin-2 junctions? Is that why only iRECs were used in this assay? Is this what has been reported in DR in vivo and that the authors have tried to recapitulate?

We thank the reviewer for this question and helpful comment. DR is characterized by a breakdown of the iBRB, resulting in decreased EC barrier function and disrupted cell-cell junctions. Interestingly, an elegant *in vivo* study found that Claudin-2 protein expression does not change in physiological and DR conditions (Luo et al., Invest Ophthalmol Vis Sci, 2011). Based on this, we hypothesized that the iRECs would respond to DR stimuli with decreased junctional protein expression (excluding Claudin-2), reduced barrier properties, and impaired vascular network integrity. We initially investigated only iRECs as we wanted to demonstrate their ability to respond to disease triggers associated with a prominent retinal microvascular disease and thus their utility for modeling retinal specific pathologies. Incorporating iECs into this testing will provide a robust non-tissue specific control that will elucidate the retinal-specific phenotype and behavior of the iRECs.

We assessed both iRECs and iECs under DR conditions. iRECs exhibited pronounced decreases in branch length, network volume, and lumen diameter, while overall surface area remained unchanged, likely due to compensatory formation of smaller, pathological vessels (Fig. 2E). Junctional proteins ZO-1, CD31, VE-Cadherin, Claudin-5, and β -catenin were significantly reduced at cell-cell contacts, confirming barrier disruption, whereas Claudin-2 expression remained stable, consistent with *in vivo* observations (New Fig. S9A). UEA1 staining revealed glycocalyx degradation in iREC networks, further recapitulating DR pathology (Fig 2Eiii).

By contrast, iEC networks were less sensitive to DR insult, showing minor changes in junctional protein expression and network morphology (New Fig. S9B-C). These findings demonstrate that iRECs not only respond robustly to diabetic stress but also faithfully capture retinal-specific vascular dysfunction, highlighting their utility as a model for DR studies.

Fig. 2E. Quantification of iREC vascular morphology with or without DR treatment

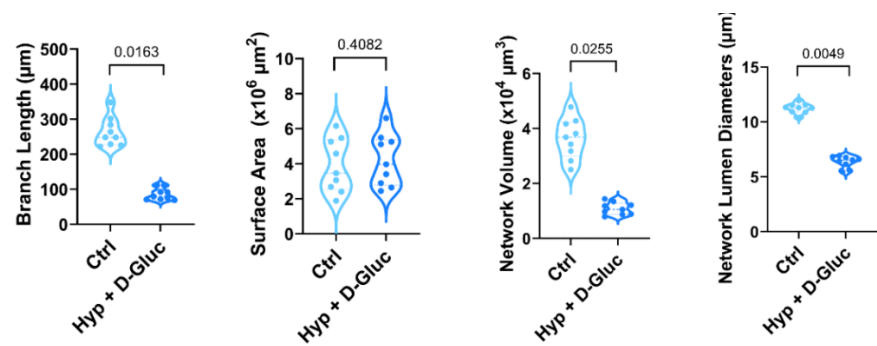


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment
Fig. 2Eiii. UEA statining in iREC vascular networks

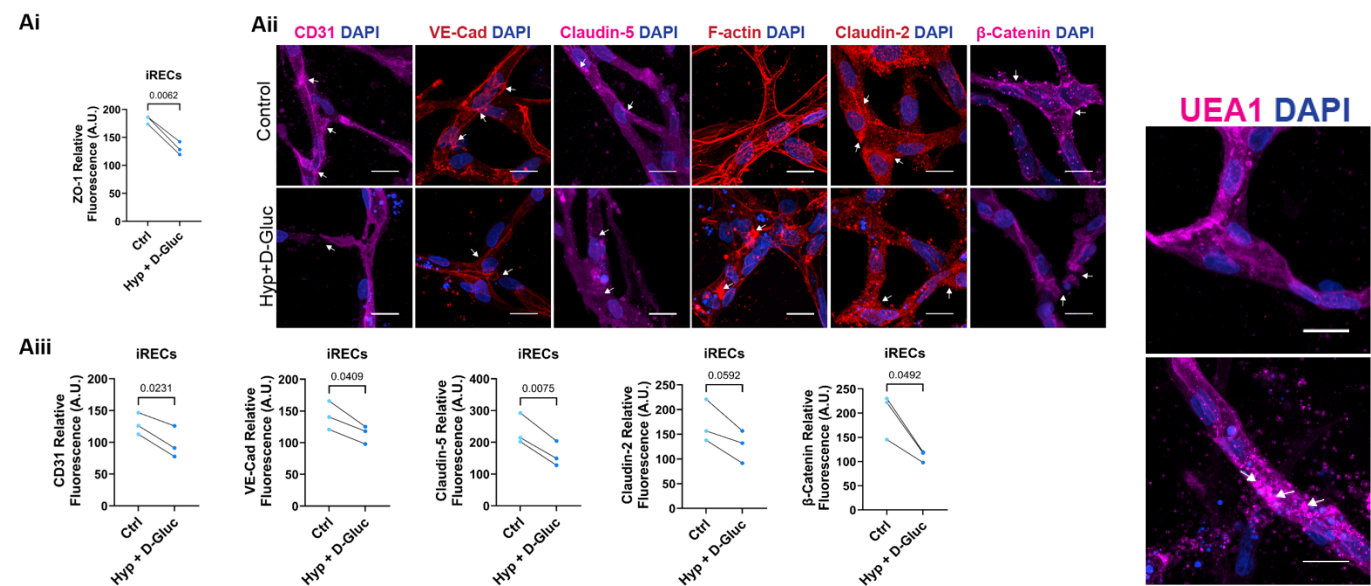
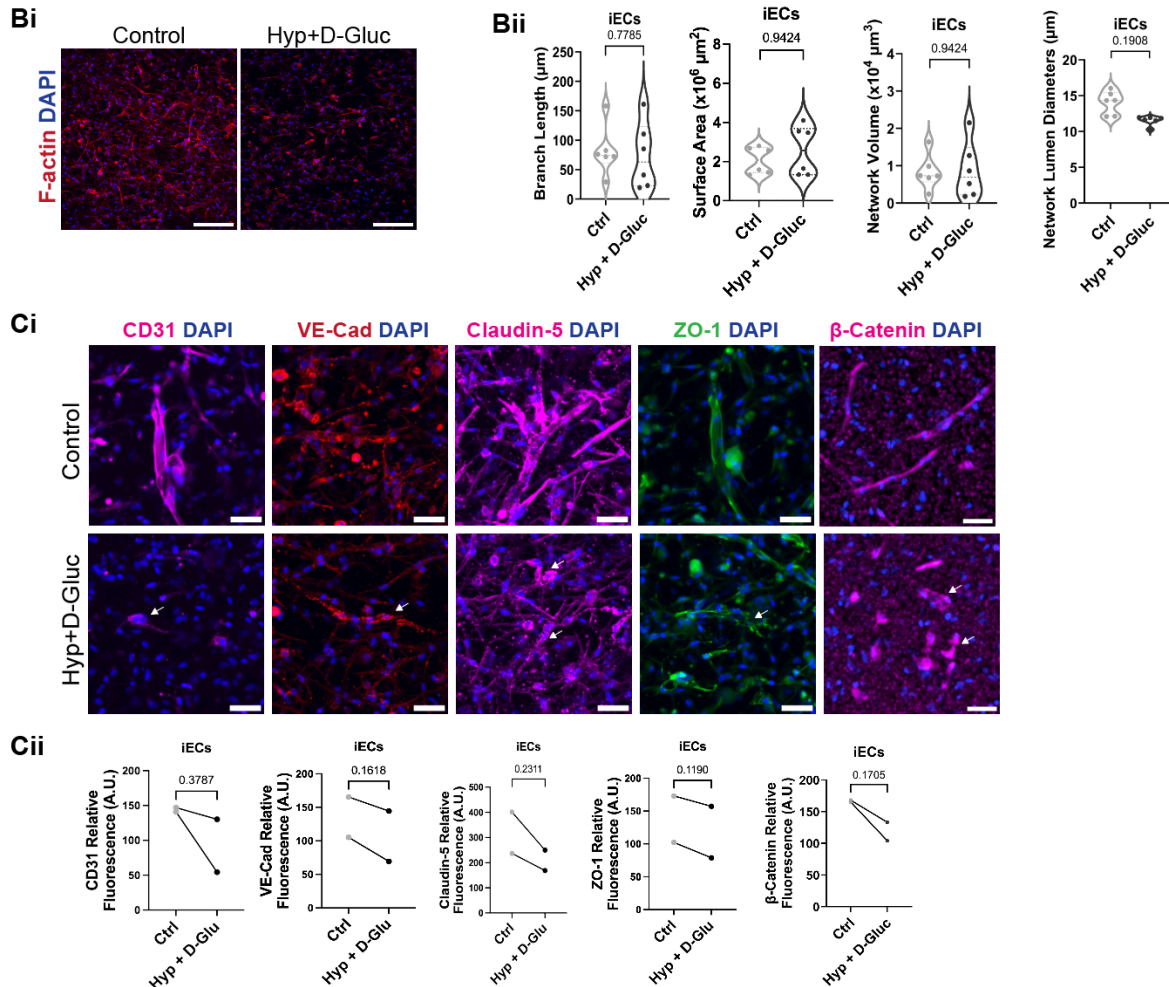


Fig. S9B. Quantification of iEC vascular morphology with or without DR treatment

Fig. S9C. 3D iEC vascular networks with or without DR treatment



9- It is not clear if that for the DR TEER assessment the cells were seeded in the same media, allowed to attach and form a monolayer and then subjected to DR model as there seem to be differences, albeit small, already at day 0.

We appreciate the reviewer's comment. We would like to clarify that both control iRECs and diabetic retinopathy-treated iRECs underwent the same experimental schedule, where they were seeded onto the Transwell on day 0 and received treatment (DR or control) on day 1. On day 0, both iEC and iRECs undergoing TEER assays were seeded on 24-well plate Transwell inserts containing 0.4 μm pores (Corning) and exposed to media composed of ECGM supplemented with 10 μM RepSox and 10% platelet poor human serum. Diabetic treatment was induced on day 1 and cells were exposed for 72-hours with media change daily (day 1 – day 4). We have updated the diabetic retinopathy treatment method section to clarify this. The first TEER measurement was made one hour after DR treatment was induced, so the slight variation in TEER values may be due to the acute impact of the DR conditions. Another possibility is that iRECs may exhibit a slower junctional maturation process compared to iECs, requiring more time to form a stable barrier. This difference in the kinetics of tight junction assembly could contribute to the minor variation observed at the early time point. Finally, The variability may also be due to the inherent variation characteristic of cells across technical replicates. Despite the apparent slight difference in TEER values on day one, post-hoc Tukey's analysis did not display any statistical difference at this timepoint.

10- Instead of comparing to iPSC-derived brain ECs, it would be more relevant to perform an unbiased scoring of the iECs and iRECs against DEGs in native RECs (which can also be obtained from published data, e.g. Menon et al Nat. Communications 2019; Watson et al Mol Vis 2020 etc.) to demonstrate which would score higher, without picking and choosing genes.

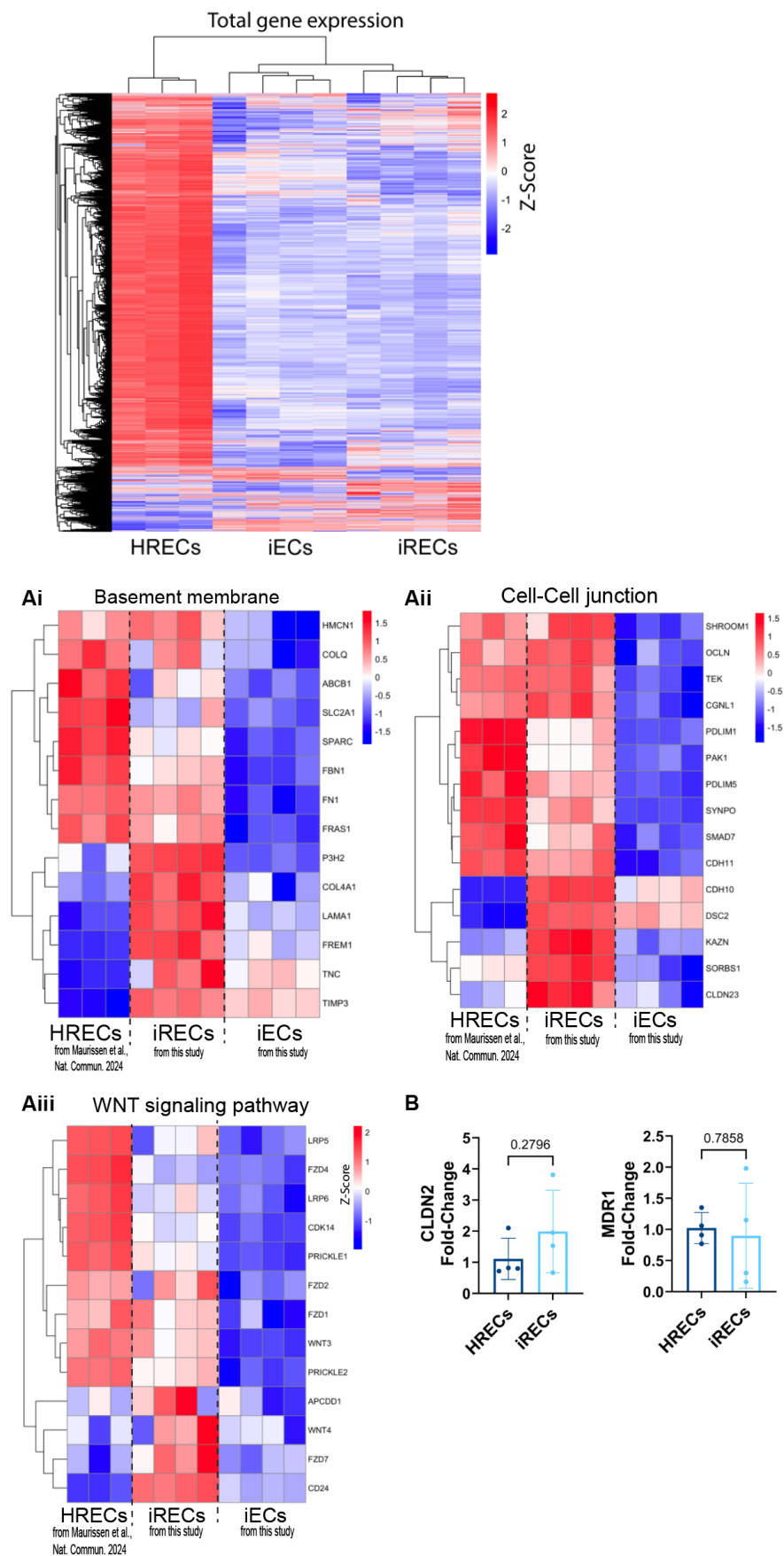
We thank the reviewer for this thoughtful suggestion. We would like to clarify that the genes used in our analysis were not selectively chosen but were derived through an unbiased approach. Specifically, we performed GO term enrichment analysis on the differentially expressed genes between iECs and iRECs to identify biologically relevant pathways. From the enriched GO categories, we then selected the top 20 most differentially expressed genes to highlight in the heat map. We better clarify this in the revised text.

We attempted to compare our data with the dataset from Menon et al. but found that it contained a limited number of retinal ECs (only 85 cells), limiting the reliability of the comparison. The dataset from Watson et al. was derived from bovine retina, which introduces species-specific differences that further complicate interpretation.

Group	Number of Cells
Rods	9145
Macroglia	5276
BPs	3155
RGCs	1514
ACs	497
Cones	211
HCs	146
Endo	85
Microglia	62

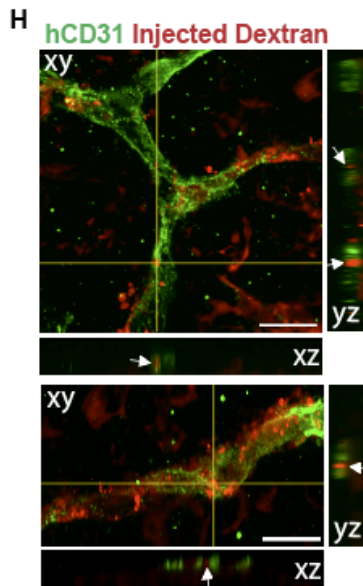
To directly assess retinal identity, we compared our iRECs to primary HRECs from Maurissen et al. (Nat Commun, 2024). Overall, iRECs display similarity to primary HRECs across multiple key gene sets relevant to retinal endothelial function (New Fig. S12A), confirming that iRECs recapitulate core transcriptional features of native retinal ECs. While the global gene expression patterns are not perfectly overlapping, this likely reflecting donor variability, culture conditions, and batch effect of RNA seq analysis.

To further substantiate these findings, we validated the retinal endothelial markers CLDN2 and MDR1 by RT-qPCR, which showed expression patterns consistent with HRECs (New Fig. S12B). Collectively, these results demonstrate that iRECs recapitulate the transcriptional and functional characteristics of human retinal ECs in a biologically meaningful way.



11- It is not clear from Fig 4C if iREC-derived vessels are perfused.

We thank the reviewer for this important comment. To verify whether the iREC-derived vessels are perfused, we administered 70 kDa rhodamine dextran via retro-orbital injection. We observed the perfusion of rhodamine dextran through human CD31 expressing iREC networks (New Fig 4H). This confirmed that the iRECs formed mature, perfusable vessels integrated with host vasculature.



12- “Corroborating these findings, a gene therapy study has reported the bilateral transfer of the substances between eyes, with visual improvement also observed in the contralateral, non-treated eye” – it would be great to have a bit more info on the ECs in the contralateral eye. Are they really cells?

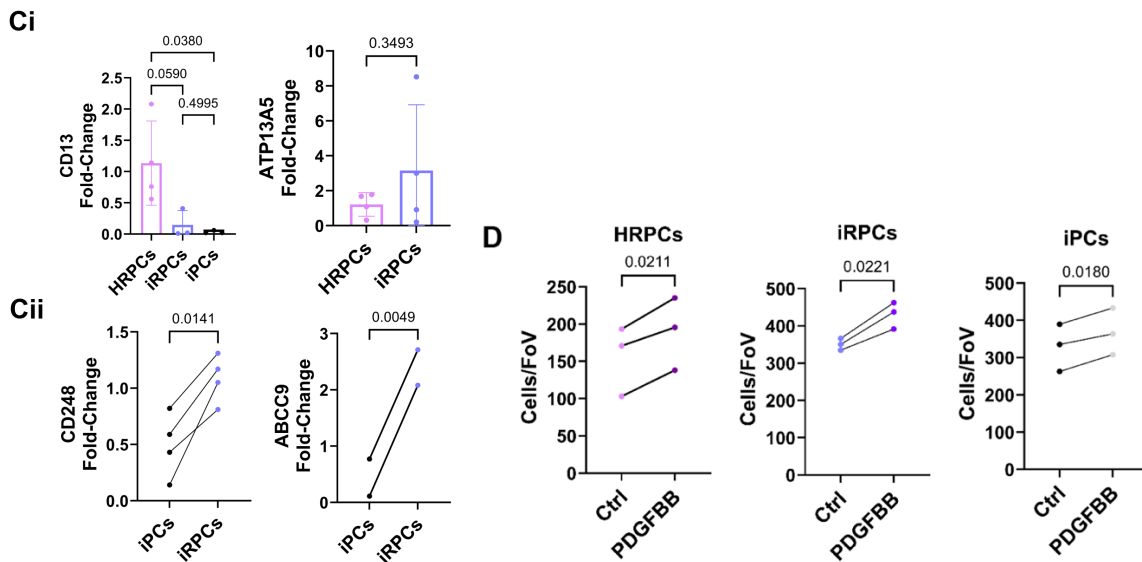
We thank the reviewer for this comment. The observation regarding potential bilateral effect was based on preliminary findings not directly related to the primary focus of this study. As our work centers on the regenerative capacity of iRECs within the treated retina, investigating whether ECs can migrate between eyes is beyond the current scope. Therefore, we have removed this statement from the manuscript to maintain focus and instead included this point in the limitations of the study.

13- Are these retina-specific pericytes or just pericytes? Very little validation is provided showing that these pericytes retina specific. Is the expression of PDGFb and Fzd4 enough to say they are retina-specific?

We thank the reviewer for this thoughtful comment. The molecular definition of retinal pericytes remains poorly established in the literature, making this a recognized limitation of the field. To evaluate the retinal specificity of our iRPCs, we compared them to human retinal pericytes (HRPCs) and non-tissue-specific iPSC-derived pericytes (iPCs) by RT-qPCR. iRPCs expressed comparable levels of the pan-pericyte marker CD13 (Fig. S16Ci) to HRPCs and iPCs, and similar levels of the retina-enriched gene ATP13A5 (Fig. S16Ci) to HRPCs. Importantly, iRPCs exhibited upregulated expression of retinal pericyte-associated markers CD248 and ABCC9 compared to iPCs (New Fig. S16Cii), suggesting a shift toward a retinal-specific pericyte identity.

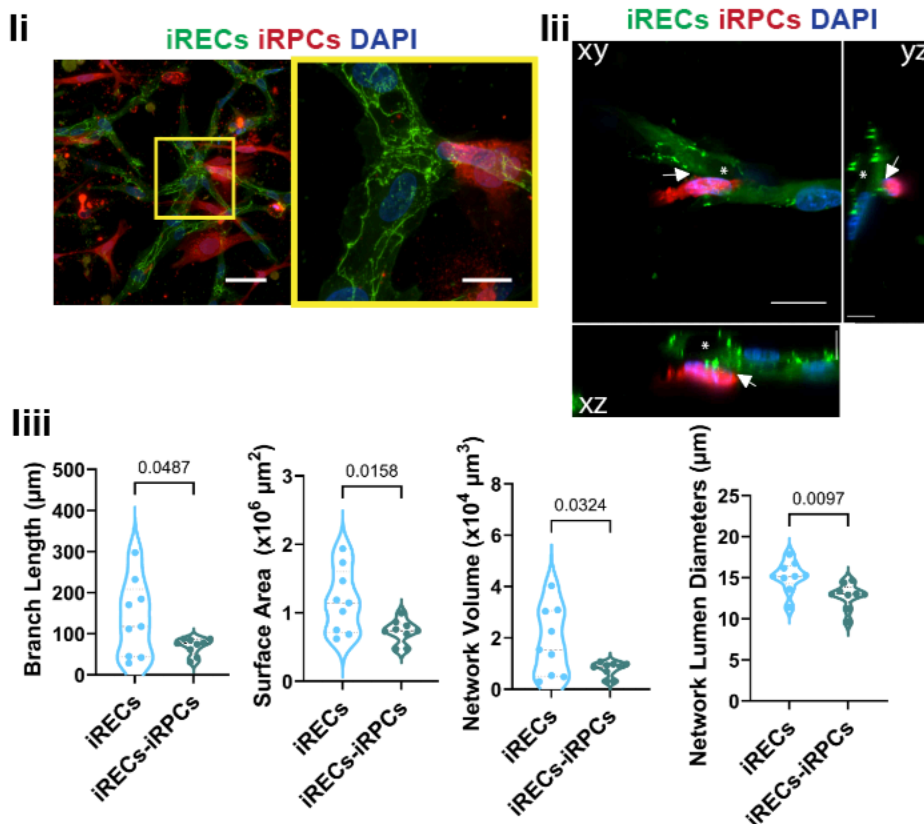
Functionally, iRPCs responded to PDGF-BB stimulation, a key endothelial-derived cue in iBRB development, through enhanced migratory capabilities within a Transwell assay. Importantly, their migration to the basolateral side of the Transwell was comparable to HRPCs and iPCs (New Fig. S16D), confirming their pericyte functionality.

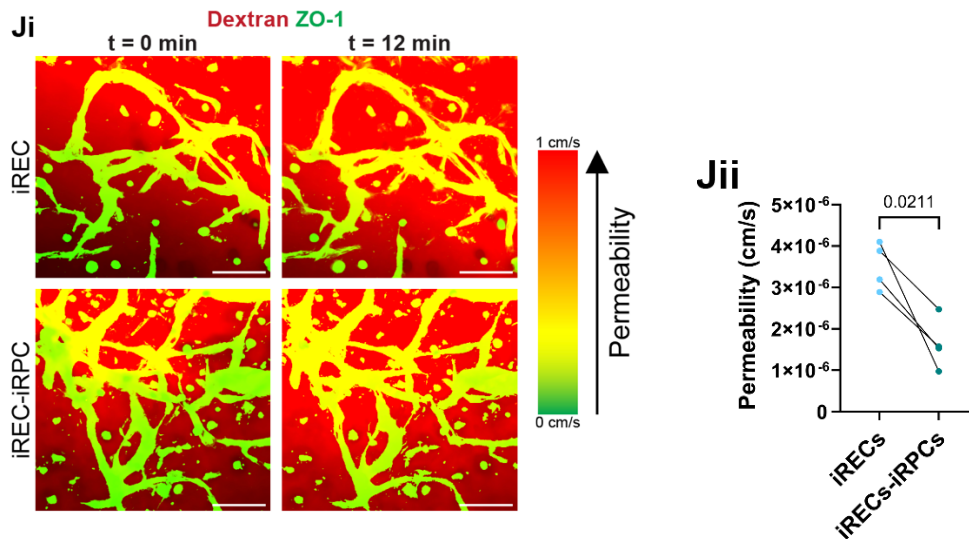
While a definitive molecular signature distinguishing retinal from non-retinal pericytes remains incomplete, our transcriptomic and functional evidence support that iRPCs acquire retina-enriched features. We acknowledge this remains an important limitation and have emphasized it in the *Limitations of the Study* section.



14- Does the inclusion of pericytes improve vessel formation, barrier function etc? This whole pericyte bit feels disconnected from the other parts of the manuscript. Perhaps using the model to study DR would be more relevant?

We thank the reviewer for bringing this to our attention. To establish the role of iRPCs within the MPS, we quantified network morphology and permeability. The presence of iRPCs within the model contributed to a decrease in vascular network branch length, surface area, volume, and lumen diameter (New **Fig. 5I**), recapitulating iBRB network morphology and the luminal size of human iBRB capillaries¹⁻⁵. The iRPCs also increased the barrier properties of the iREC networks, decreasing their 70 kDa rhodamine dextran permeability to a level consistent with *in vivo* permeability (New **Fig. 5J**).





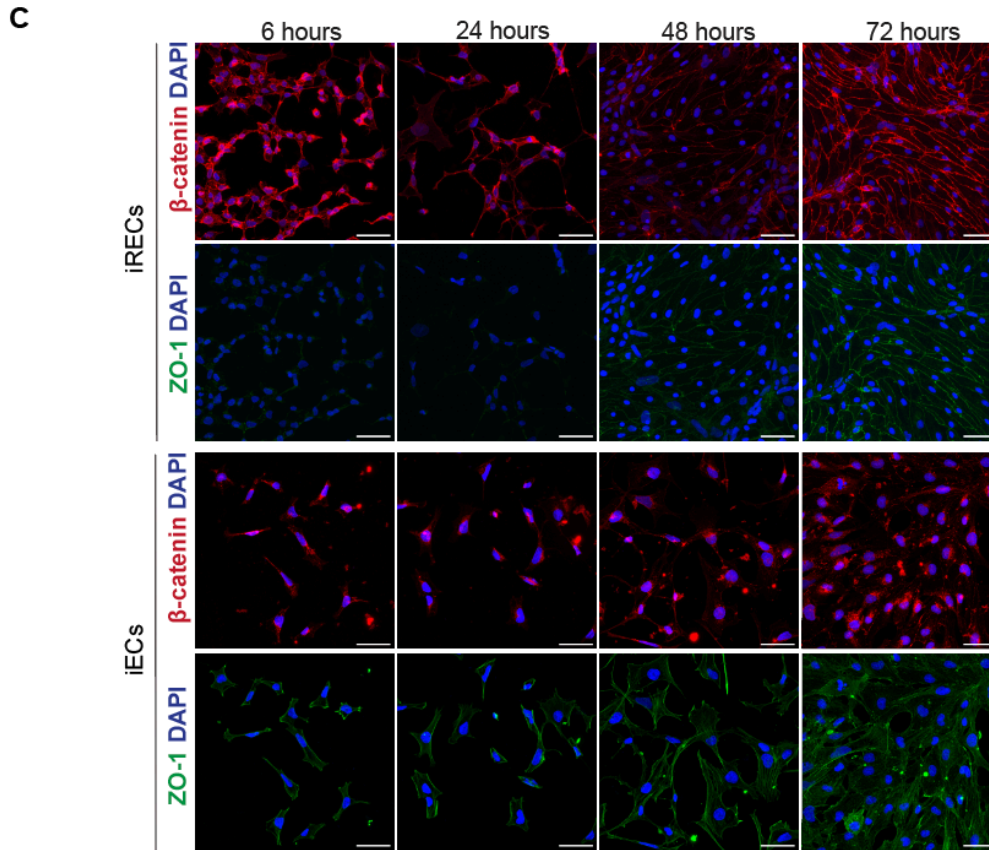
Minor criticism

1. Fig S1 caption reads: “and functional analysis” but there is no functional analysis reported.

We thank the reviewer for pointing this out. We have updated the caption of Fig S1 to remove “functional analysis”.

2. Panel C should also show generic ECs.

We thank the reviewer for this helpful comment. We have now included iECs in New Fig. S2C for direct comparison. We observed that iECs exhibited minimal β -catenin junctional localization, whereas in iRECs, substantial β -catenin translocated from the cytoplasm to cell–cell junctions within 48 hours after ETV2 induction. These findings demonstrate that Norrin-mediated Wnt/ β -catenin signaling is functionally activated during iREC specification, supporting the conclusion that iRECs recapitulate key aspects of retinal endothelial development and identity.



3. Fig S2 would benefit from quantitative analysis

We thank the reviewer for this suggestion. We have now included quantitative fluorescence analysis of the 3D vascular networks formed by iECs and iRECs (New Fig. S7). The results show significantly higher fluorescence intensity of junctional proteins ZO-1, Claudin-2, and Claudin-5 in iRECs, while canonical endothelial markers (CD31, VE-Cadherin, UEA1) remained comparable between the two cell types. These quantifications strengthen the conclusion that iRECs exhibit enhanced junctional organization consistent with a retinal-specific endothelial phenotype.

4. iBMEC – acronyms should be defined at first appearance

We thank the reviewer for pointing this out. The definition of iBMEC is now provided in the figure legend of New Fig. S13.

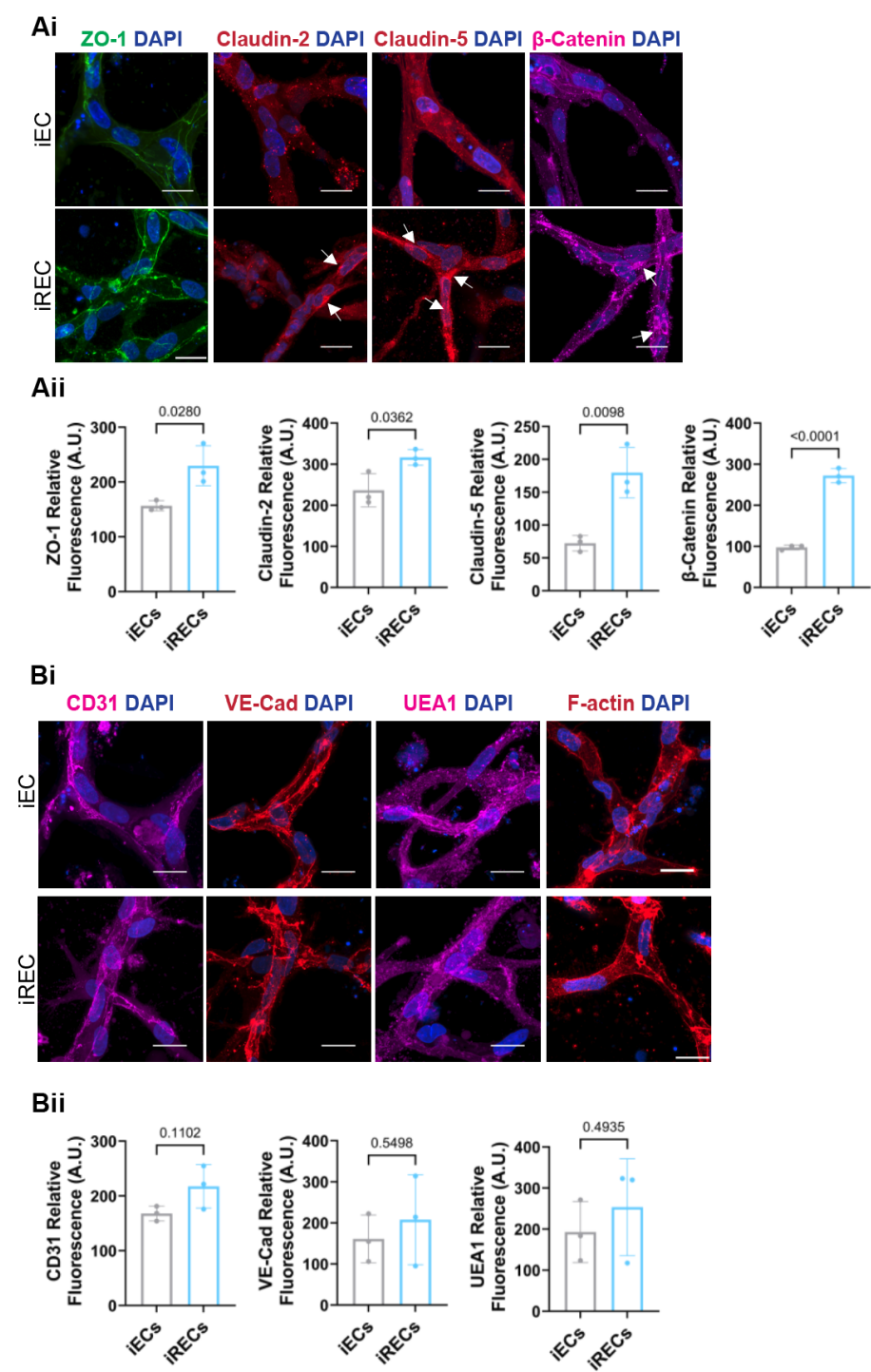
5. Fig 2 Ci could be moved to supplemental as it does not add critical info

“Specifically, the iREC networks contain heightened membrane-bound Claudin-2 and Claudin-5 cell-cell contact expression relative to iEC networks (Fig S7A)” there is no quantification to allow this statement. Claudin -5 to me looks the same. Providing only 1 image without quantification demonstrate lack of rigor.

We thank the reviewer for this valuable comment. We have updated the figure (New Fig. S7) to provide more accurate representative figures and include quantitative analysis of fluorescence intensity for junctional markers, and revised the text accordingly.

The iREC networks exhibited higher overall fluorescence intensity of ZO-1, Claudin-2, Claudin-5, and β -catenin compared to iEC networks (New Fig. S7A), consistent with their more continuous junctional distribution observed in immunostaining. In contrast, expression of canonical endothelial markers (CD31, VE-Cadherin,

UEA1) were comparable between iREC and iEC networks (New Fig. S7B), confirming that both maintain endothelial identity.



6. Provide lower magnification images for Fig 5 Dii and assessment of network perfusion

We thank the reviewer for the suggestion. Lower magnification images have been added as supplementary Figure 15Eii.

7. Missing or unclear details about statistics, protocols or materials

We thank the reviewer for pointing this out. We have updated the method section to include additional details on protocols, cell numbers, experimental timeline, statistics, and materials used.

8. For quantifications (e.g. Fig 2Dii) authors need to provide details of what was assessed, were all points measured from the same assays or 8 different biological replicates (differentiations and different runs)? In other words, are these technical replicates of real separate assays?

We thank the reviewer for bringing this to our attention. The quantification in Fig 2Dii was performed using three independent biological replicates, each containing three technical replicates (total of nine data points shown in the graph). For each technical replicate, ten cells were quantified to obtain the fluorescence intensity values for the entire sample. We included all the datapoints within the figure to enable easy visualization of the results. We have updated figure legends and the methods section to clarify our quantification. We have updated figure legends and the methods section to clarify our quantification for all experiments.

Reviewer #2 (Report for the authors (Required)):

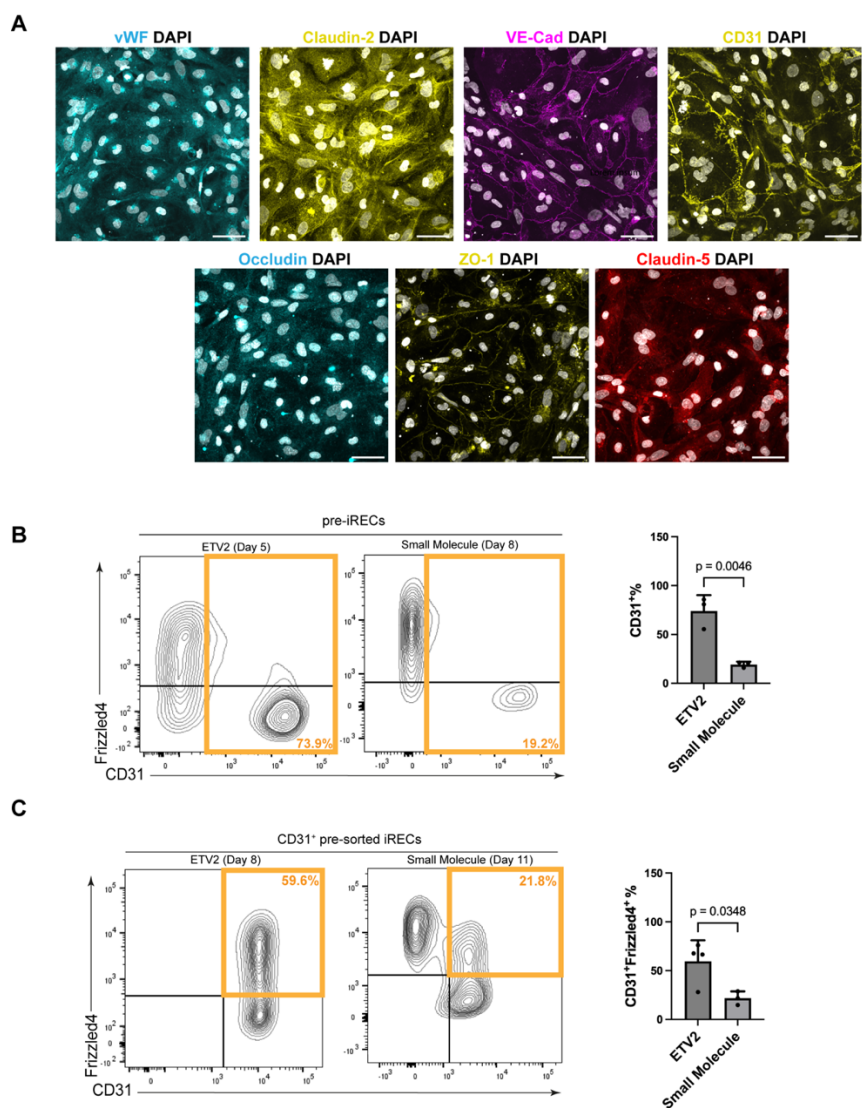
The reviewed paper aims to modify the existing differentiation protocol of endothelial cells (ECs) and pericytes (PCs) from iPSCs to mimic the retinal EC and PC phenotypes. Although the proposed project possesses high impact due to providing materials for otherwise hardly accessible cell types for disease modeling and therapeutic approaches, the current state of the project cannot yet be considered complete.

(1) The authors modified the existing protocol for electroporation-induced EC differentiation to drive additionally treating with Norrin and vitronectin to induce the retinal EC (iRECs). As a result the cell population iREC positive for CD31 and Fz4 was received after two rounds of cell sorting. For the iREC induction protocol, I have the following questions:

Why was the electroporation protocol chosen as a base protocol? Did the authors try standard EC induction with growth factors and it didn't work or was the electroporation protocol the first choice? Electroporation has limitations for possible therapeutic usage: although a non-integrative method was used, it still increases the risk for genomic instability.

We thank the reviewer for this important point. We evaluated the iREC small-molecule differentiation protocol (New Fig. S5) and found that, while it successfully generated iREC expressing functional iREC markers, the overall efficiency and yield of CD31⁺ cells were significantly lower compared to the ETV2 electroporation-based method (19.2% vs. 73.9%). Specifically, the CD31⁺Fz4⁺ iREC population pre-Fz4 sorting was significantly reduced using the small molecule approach (21.8% vs. 59.6%). Based on these findings, we selected the ETV2 electroporation protocol as our base method to ensure robust production of iRECs. ETV2-based protocols have generated biologically accurate ECs^{6,7}, supporting our utilization of this technique.

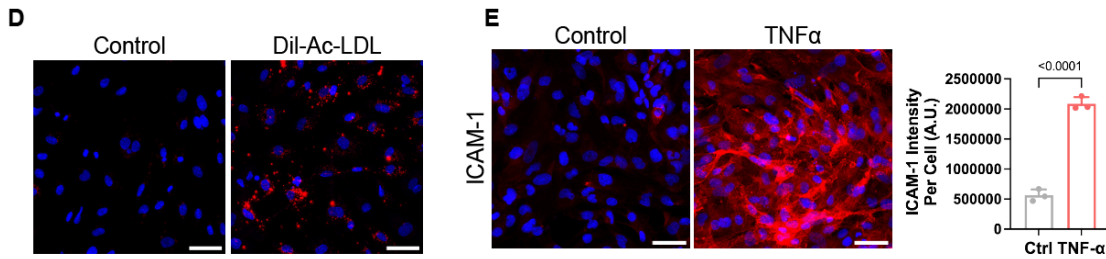
We acknowledge that electroporation may increase the risk of genomic instability, which is an important consideration for therapeutic applications. In this study, electroporation was selected for its efficiency and reproducibility in generating high yields of CD31⁺Fz4⁺ iRECs population. At the same time, alternative strategies for ETV2 induction have been reported, including stable doxycycline-inducible iPSC lines (Gong et. al., CSC, 2025), and viral-mediated delivery (Morita et. al., PNAS, 2014). An emerging mRNA delivery technology based on mechanical disruption (Stewart and Sharei et. al., Nature, 2016) represents a promising alternative to induce transient gene expression with reduced genomic risk. This approach could be further explored in future studies to improve the clinical applicability of iREC generation.



Were there any functional assays done to prove the EC differentiation? Several previous attempts to receive brain ECs phenotype resulted instead in the differentiation of epithelial-like cells then ECs. Therefore it is essential to verify the EC identity of iRECs with functional assays, such as (1) an actively take-up acetylated LDL and (2) ICAM expression upon TNF- α treatment. These additional experiments would confirm that ECs preserved cell identity after Norrin and vitronectin induction.

We thank the reviewer for this insightful comment. We have confirmed the endothelial identity of iRECs through immunofluorescence imaging and flow cytometry which showed robust expression of endothelial markers including CD31 (Fig. S2A, and Fig. 1Ci-iv), vWF (Fig. 1D), and VE-cadherin (Fig. 1D). In addition, iRECs demonstrated endothelial functionality by forming 3D lumen-like structures with comparable branch length and surface area to iECs in 3D collagen type-I hydrogels (Fig. 2Ci) and within the iBRB-on-chip system (New Fig S15Bii and Ciii).

Furthermore, iRECs actively internalized Dil-acetylated LDL and responded to TNF α stimulation with increased ICAM-1 expression (New Fig. S2D-E), confirming that they maintain hallmark endothelial functions.



(2) The authors used the expression of two cell markers, CD31 and Fz4, as hallmarks of retinal EC and PC specification. However, both iECs and pre-iRECs were shown to express CD31 and Fz4. iREC induction was performed by adding the Fz4 ligand, Norrin. The efficiency of iREC differentiation was assessed by the increase in the Fz4⁺ cell population. But isn't the most obvious explanation for this increase a positive feedback loop, where Norrin induces the expression of its receptor, Fz4? If so, the results may reflect Norrin's effect on ECs rather than the emergence of a distinct iREC population.

We thank the reviewer for raising this important point. While it ligand exposure could potentially influence receptor expression, to our knowledge, there is no literature evidence supporting a positive feedback loop between Norrin and Fz4.

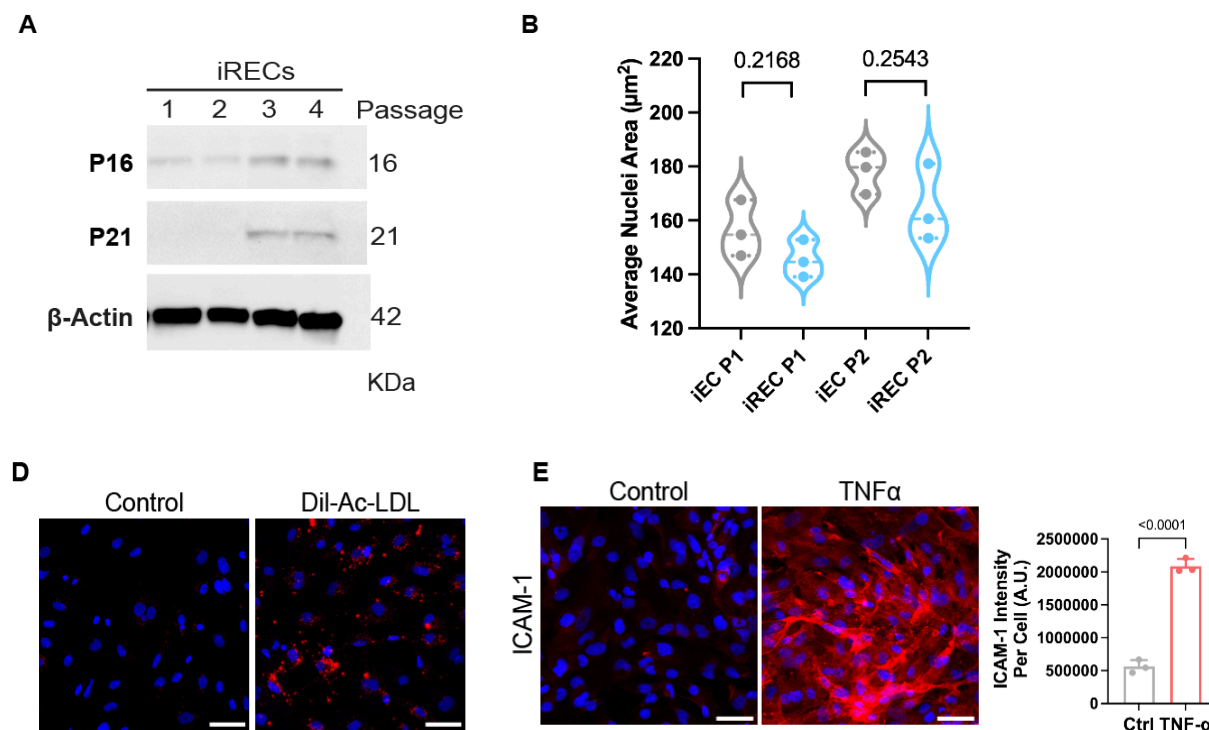
The CD31⁺ pre-iRECs were cultured in the absence of Norrin from day 5 to day 8, using endothelial cell growth medium (ECGM) supplemented with 10 μM RepSox and 10% platelet poor human serum. On day 5, 58.3% of CD31⁺ pre-iREC expressed Fz4, but this portion increased to 66.5% by day 8, despite the removal of Norrin. This suggests that the increase in Fz4 expression is not likely a direct result of ongoing ligand stimulation rather a progression in cell identity toward an iREC phenotype.

Furthermore, iRECs exhibit hallmark retinal endothelial characteristics, including higher TEER and active GLUT1-mediated glucose transport (Fig. 2A – B), and display a distinct transcriptomic profile from iECs (Fig. 3), confirming that they represent a specialized retinal EC population rather than a Norrin-stimulated iEC variant.

(3) Figure 1D demonstrates the differences in morphology and marker expression of iECs and iREC. The morphology of iECs appears healthier: cells are uniformly shaped and properly aligned with each other. The round and irregular shape of iRECs, on the other hand, reminds features of senescence or dysfunctional ECs.

We thank the reviewer for this insightful comment. To address concerns regarding potential senescence, we performed western blot analysis for cyclin-dependent kinase inhibitors p21 and p16 that induce senescent cell-cycle arrest across multiple iREC passages (New Fig. S4A). Senescence markers was upregulated beginning at passage 3 and therefore all experiments were conducted using passage 1-2 iRECs, where no significant upregulation of senescence markers was detected. We also compared the nuclear area of iECs and iRECs between passages 1-2 and found no significant difference (New Fig. S4B). This suggest that the distinct morphology and marker expression of iRECs likely reflects inherent phenotypic difference from iECs rather than senescence-associated changes.

To further verify that iRECs are functional ECs, we conducted functional assays and found that iRECs actively internalized Dil-acetylated LDL and responded to TNFα stimulation with increased ICAM-1 expression (New Fig. S2D-E), demonstrating that they are mature, functional ECs.



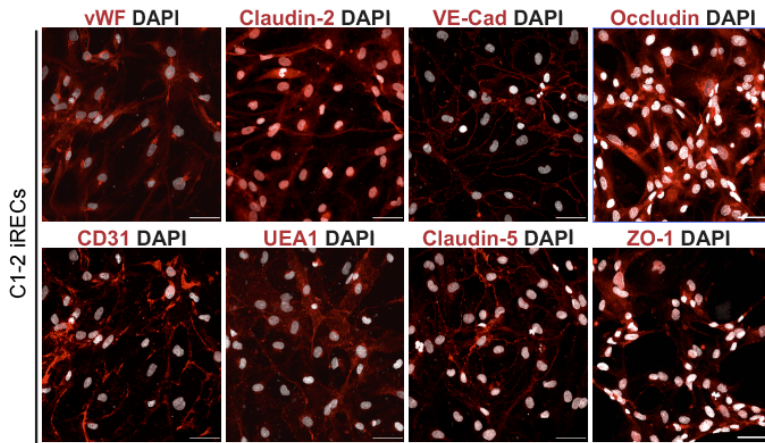
(4) Figure S2 demonstrates the staining and morphology of HRECs. The cell density appears low and cell morphology is generally unhealthy. Authors should consider excluding these cells from the analysis since results received with unhealthy cells are unreliable.

We thank the reviewer for this insightful comment. We obtained primary HRECs directly from Cell Systems and used them only between passage 4-6. Within these passages we did not observe the loss of endothelial marker expression or significant morphological abnormalities that would suggest compromised cell health. Moreover, HRECs from the same supplier and within comparable passage ranges were used in our previous study (Cho and Macklin et al., JCI Insight, 2020), where they exhibited similar morphology and EC marker expression. Based on these considerations, we are confident that the HRECs used in this study were healthy and suitable for analysis.

(5) Figure S2C should prove the robustness of the protocol by using another hiPSC line to differentiate iRECs. However, the authors do not show the expression of ZO-1 and Occludin, two of the cell junction markers, they claimed to be increased by this protocol compared to iECs.

We thank the reviewer for this comment. We have included immunofluorescence staining of ZO-1 and Occludin for C1-2 iRECs.

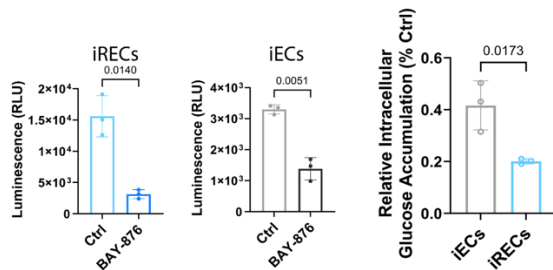
C



(6) Authors demonstrate that iRECs have a higher TEER than iECs. However, they do not provide the data on Bay-876 treatment. Bay-876 treatment significantly reduces the glucose uptake due to GLUT1 inhibition of iRECs, but does it have the same effect on iECs?

It is crucial to assess the GLUT1 transport in both cell types if the glucose transport serves as a retinal endothelial phenotype.

We thank the reviewer for this important comment. We have now included iECs in the GLUT1 transport assay (New Fig. S6C). GLUT1 is the primary facilitator of glucose transport, a key feature of retinal endothelial cells. At baseline, iRECs exhibited ~5-fold higher glucose uptake than iECs (1.5×10^4 vs. 3×10^3 ; Fig. 2B, New Fig. S6Ci). Treatment with the selective GLUT1 inhibitor Bay-876 reduced glucose uptake by 80% in iRECs but only 60% in iECs (New Fig. S6Cii). These results demonstrate that iRECs possess higher GLUT1 activity and transporter functionality, reinforcing their retinal-specific metabolic phenotype.



(7) The authors claim in Figure S3A, that iRECs show increased Claudin-2 expression at cell-cell junctions compared to iECs. Later, in Figure S4C, they claim that the Claudin-2 expression of iRECs in diabetic conditions does not differ from the control. However, the shown representative images of Claudin-2 staining in iECs and diabetic iRECs look very similar. So either there is no Claudin-2 expression difference between iECs and iRECs, or the diabetic treatment of iRECs caused a reduced localization of Claudin-2 at cell-cell junctions.

We appreciate the reviewer's comment. To address this concern, we provided more accurate representative figures and quantified the total fluorescence intensity of Claudin-2 expression in both iEC and iREC vascular networks (New Fig. S7A). This analysis confirmed that iRECs exhibit higher Claudin-2 expression compared to iECs. Similarly, we provided new representative figures for iRECs treated with DR and quantified their Claudin-2 protein expression (New Fig. S9Aii-iii). Claudin-2 expression was consistent between iRECs treated with and without DR condition.

Fig. S7A. 3D iEC and iREC vascular networks

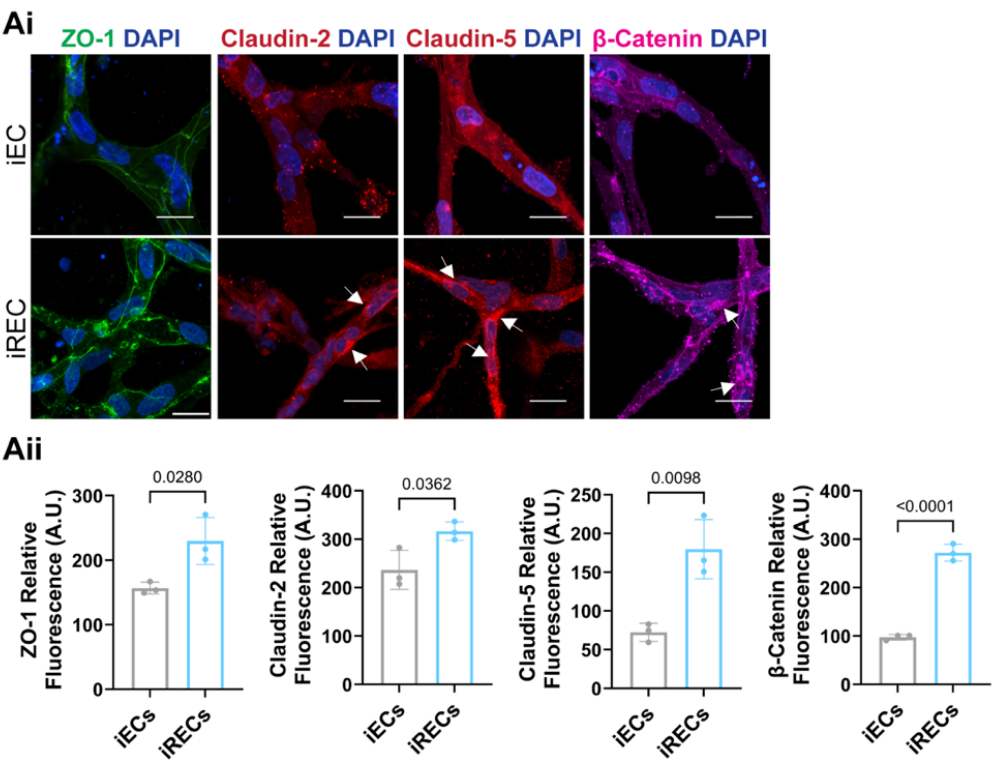


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment

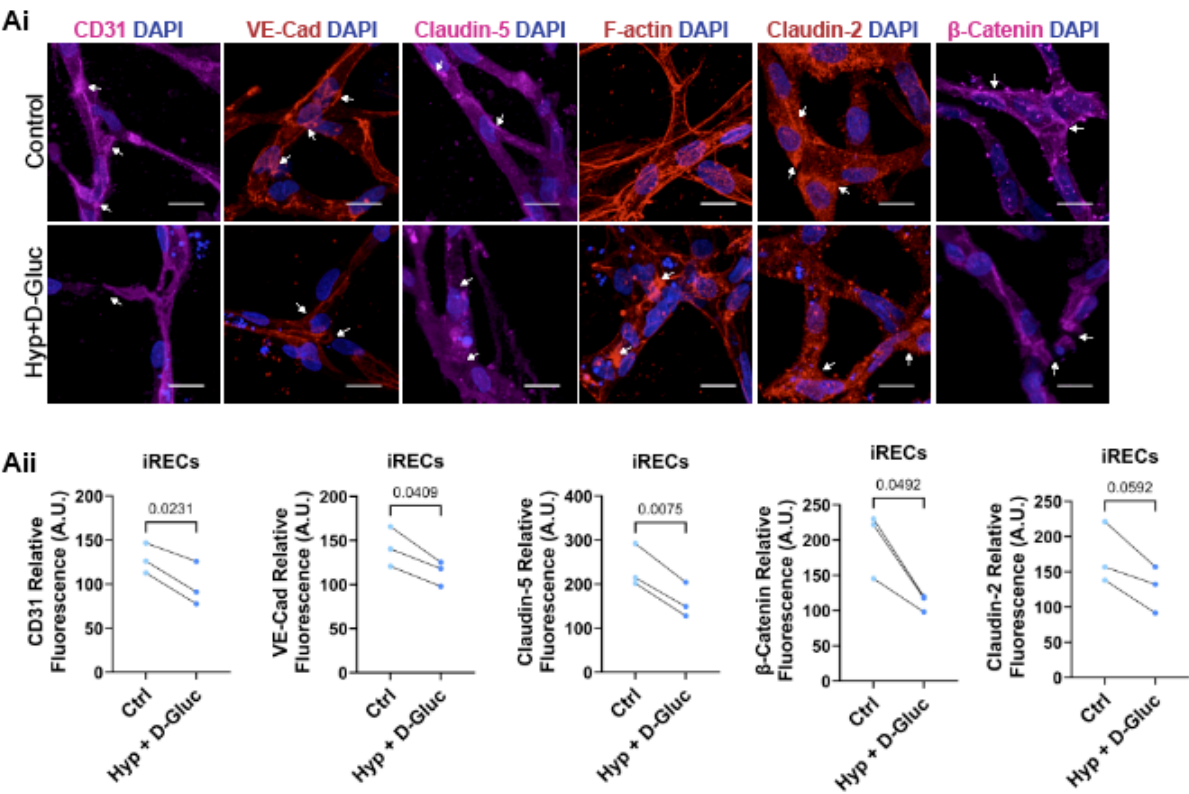
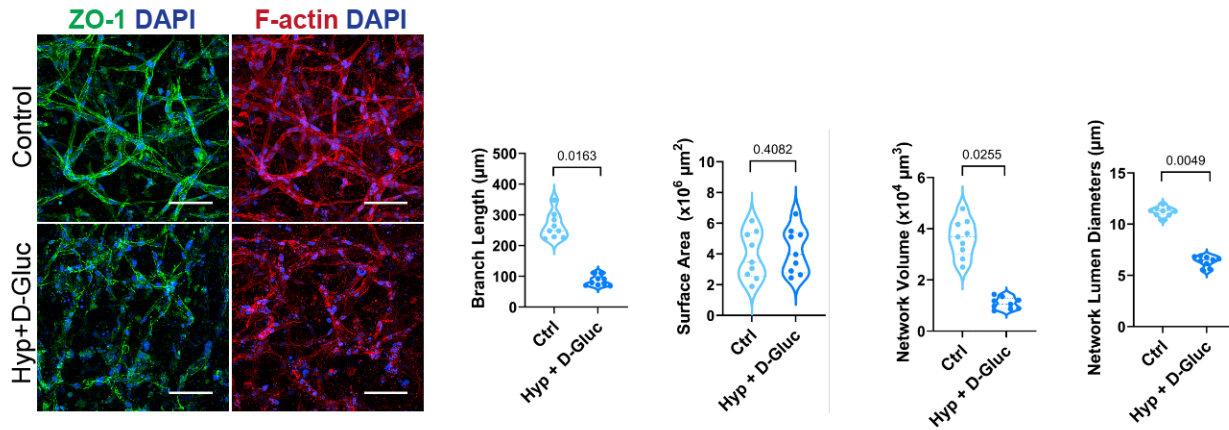


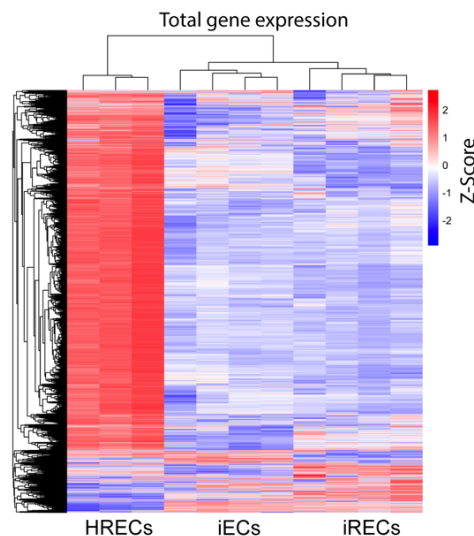
Fig. 2E. 3D iREC vascular networks with or without diabetic treatment and morphology quantification

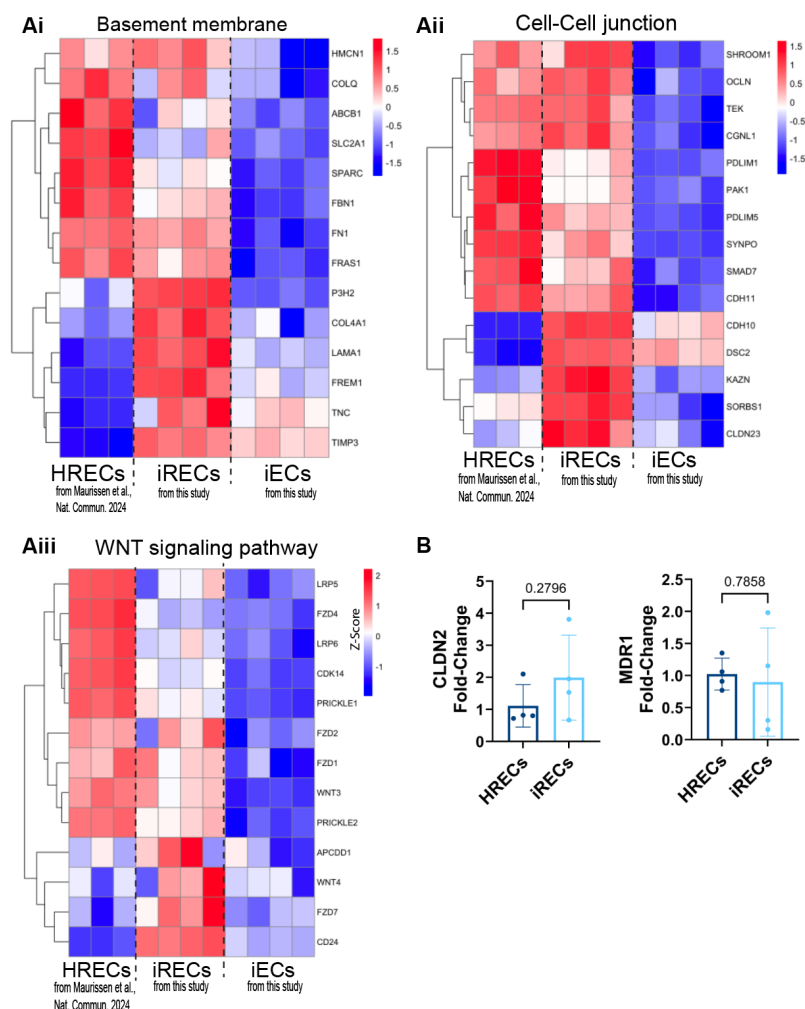


(8) The authors do a transcriptomic analysis of iRECs, and iECs and compare them with iPSC-derived brain ECs. Of course, there are similarities (both ECs) and differences (different differentiation protocols). Would it not have been more meaningful to compare the iRECs and iECs with human retinal endothelial cells, since the focus of the paper is to prove that iRECs show more retinal identity than iECs?

We thank the reviewer for this important comment. We initially did not compare iRECs with Primary HRECs due to concerns that the primary cells lost tissue-specific phenotypes when cultured *ex vivo*. As a result, we originally compared the iRECs to iECs as they have been previously validated and their robustness has been confirmed.

To address reviewers suggestion, we compared our samples to primary HRECs from Maurissen et al. (Nat Commun, 2024). Importantly, we observed similar expression between our iRECs and human primary retinal ECs across multiple relevant gene sets (New Fig. S12A). While the global gene expression heat map does not show perfect similarity, this may partly reflect the inherent variability among primary cell donors, differences in culture conditions, or batch-related confounding factors. These limitations highlight the need of iPSC-derived retina ECs as a more consistent and reproducible model. To further strengthen this comparison, we performed RT-qPCR validation of the retinal endothelial markers, CLDN2 and MDR1, confirming iREC expression patterns similar to HRECs (New Fig. S12B).





(9) Revascularization experiments in the ischemic eye were conducted only with iRECs. To demonstrate the increased therapeutic potential of iRECs, it is important to compare them with iECs. Ideally, the therapeutic effect of iRECs should also be compared with that of primary mouse retinal endothelial cells.

We thank the reviewer for this comment. To directly compare therapeutic efficacy, we included iECs as a control in the OIR model. Consistent with prior reports, iEC treatment reduced neovascularization (NV) and vaso-obliteration (VO) relative to PBS controls (New Fig. S14A). Both iECs and iRECs integrated with host vasculature and reduced vascular leakage (New Fig. S14B, S14F, 4C, 4G). Functional perfusion of iREC-derived lumen-like structures with rhodamine-dextran confirmed successful vascular integration and maturation (New Fig. 4H). Notably, iRECs demonstrated better efficacy than iECs, restoring physiological vascular network with smaller segment volumes and lumen diameters while iECs did not impact these parameters (Fig. 4E and New Fig. S14D). These results emphasize the therapeutic and biological significance of retinal-specific ECs.

Primary mouse retinal ECs were not included due to species-specific differences that could confound interpretation. Comparison with non-retinal iECs provides a relevant benchmark, highlighting the enhanced retina-specific reparative potential of iRECs.

Fig. S14A-B. iECs injected OIR retina

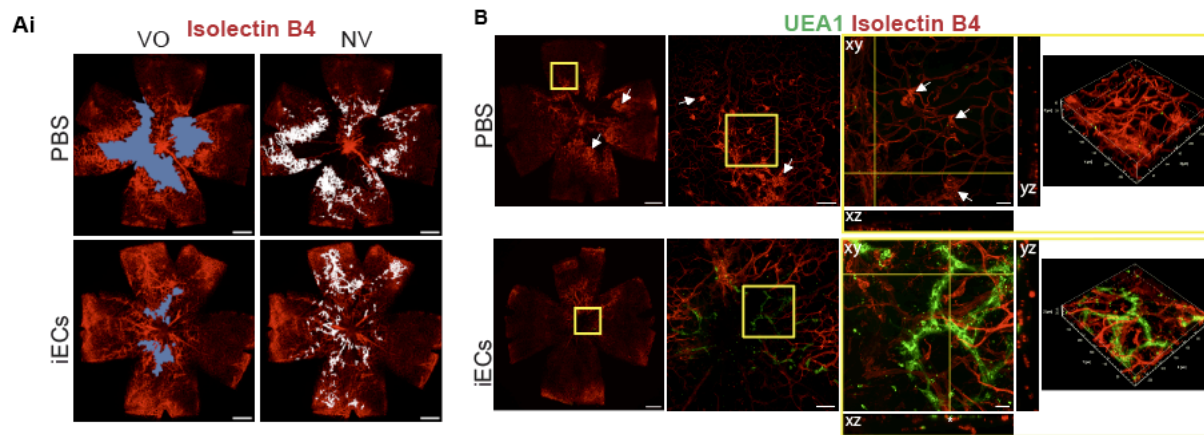


Fig 4E. Quantification of mouse vascular network segment volume and lumen diameter across PBS and iREC injected groups

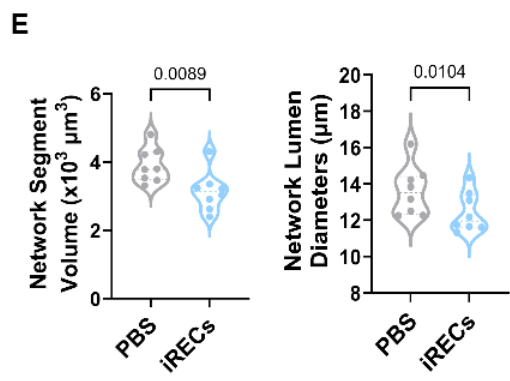


Fig. S14F. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iEC injected OIR retinas

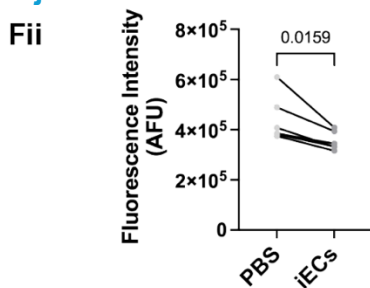


Fig.4C iRECs injected OIR retina.

Fig.4Gii. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iREC injected OIR retinas.

Fig. 4H. iREC networks perfused by injected 70 kDa rhodamine dextran within OIR retinas

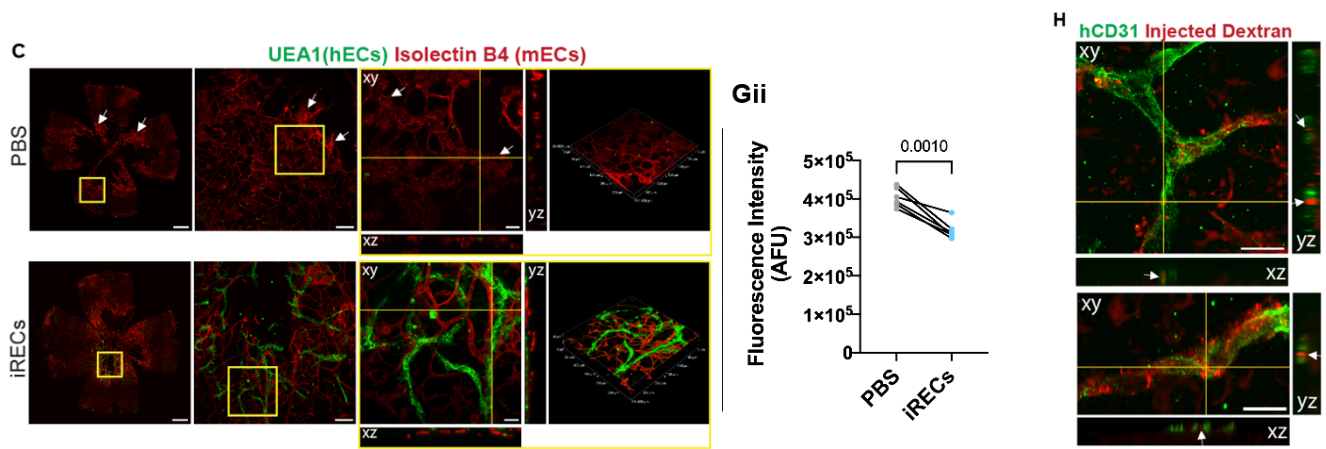
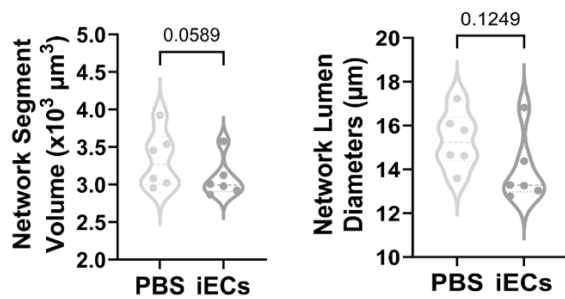
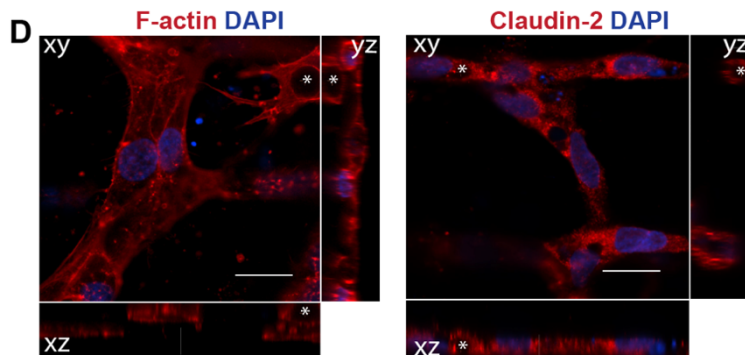


Fig 4E. Quantification of mouse vascular network segment volume and lumen diameter across PBS and iEC injected groups



(10) In Figure 5Di, the authors show the existence of a lumen in the vasculature. However it seems, that not every asterisk is indeed a lumen, some might be just holes in the vessels (especially the ones shown in the xy-image in which you can not prove that the lumen is enclosed by ECs).

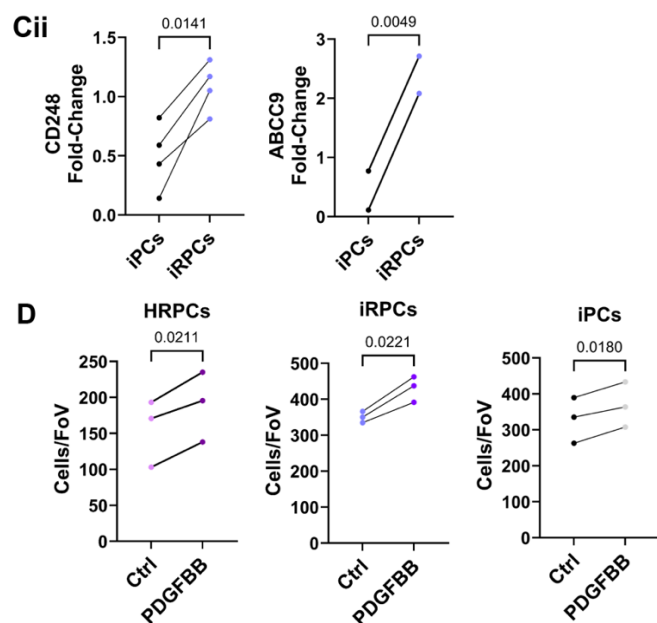
We thank the reviewer for pointing this out. We have revised Fig. 5D to ensure that asterisks indicate only lumens enclosed by ECs. We specifically selected lumens that are supported by ECs that can be visualized in the xy-, yz-, and/or xz-plane(s).



(11) Are there any differences in marker expression between mesodermal PCs and iRPCs? The effect of increased Fz4 could be the same as in iRECs: the introduction of the ligand Norrin may upregulate Fz4 expression through a positive feedback loop, rather than proving the existence of a new cell subpopulation.

We thank the reviewer for this insightful comment. iRPCs were exposed to Norrin only during the six day differentiation phase and subsequently cultured in Pericyte Medium without Norrin from passage 1 through passage 10. This long-term maintenance without Norrin indicates that the sustained FZD4 expression reflects stable retinal pericyte specification rather than a transient positive feedback effect.

To further substantiate retinal identity, we performed qPCR analysis of retinal pericyte-specific markers, CD248 and ABCC9, which showed significantly higher expression in iRPCs compared to non-retinal iPCs (New Fig. S16C). Functionally, iRPCs demonstrated robust migration in response to PDGF-BB, confirming canonical pericyte competence (New Fig. S16D). While we acknowledge that retinal pericyte characterization remains limited in the literature, these results provide strong evidence of iRPC lineage fidelity and functional capacity, as discussed in the “Limitations of the study” section.



(12) The Permeability assay is missing in the methods.

We thank the reviewer for pointing this out. We have updated the methods section to include a detailed description of the permeability assay.

(13) The used iPSC line contains EGFP-tagged ZO-1. However, the images are still referred to as immunofluorescence of ZO-1. Were these cells additionally stained with antibodies against ZO-1?

We thank the reviewer for pointing this out. The hiPSC line used was the mEGFP-tagged TJP1 WTC line obtained from the Coriell Institute. We did not stain these cells with an additional antibody against ZO-1. The only cells we stained for ZO-1 utilizing a primary antibody was the C1-2 hiPSC and primary HREC lines as they were not tagged. We have updated ZO-1 to ZO-1-GFP to reflect the EGFP-tagged signal in the figure legends throughout the manuscript.

In summary, several critical issues need to be addressed before the paper can be considered for publication. The presented results might be interpreted as the Norrin impact on gene expression, TEER, and microvasculature formation, but they are not proof of successful iREC/PC differentiation protocol. Due to multiple methodological inconsistencies and a lack of results on multiple cell lines, I can not recommend the paper for publication.

We thank the reviewer for their thoughtful comments and recognition of the potential impact of our work. We have addressed all the concerns raised with new data that strengthen the rigor, clarity, and novelty of our findings.

Reviewer #3 (Report for the authors (Required)):

This manuscript describes a method to derive human retinal endothelial cells (iRECs) from hiPSCs by activating the Wnt/ β -catenin signaling pathway, specifically through Norrin/Fz4 signaling. The authors claim that these iRECs recapitulate key molecular, phenotypic, and functional features of native retinal endothelial cells, including barrier function, responsiveness to diabetic retinopathy (DR)-like conditions, and in vivo vasculogenic potential in an oxygen-induced retinopathy (OIR) model. They further demonstrate the integration of iRECs with iPSC-derived retinal pericytes to form a perfusable, microphysiological iBRB-on-a-chip platform. This work is positioned within the fields of vascular biology, regenerative medicine, and ophthalmology, where robust, tissue-specific endothelial cell models are critical for disease modeling and therapeutic development.

The development of protocols aimed at generating endothelial cells with bona fide tissue-specific identities is an area of growing importance in vascular biology, with significant implications for both mechanistic studies and translational applications. In this context, the authors' efforts to define and validate a retinal-specific endothelial phenotype from hiPSCs address a timely and relevant question. The manuscript presents a technically ambitious and well-structured body of work, and the integration of iRECs into a microphysiological model is a notable strength.

However, based on the current data, the study does not yet establish that iRECs have acquired a definitive retina-specific identity that functionally distinguishes them from other iPSC-derived endothelial cells. Key aspects of tissue specificity—particularly at the transcriptomic, functional, and in vivo levels—would benefit from further support.

I have the following major concerns.

Major Concerns

1. The study does not provide an adequate dissection of the differentiation protocol components. The iREC protocol relies on sequential exposure to Norrin and vitronectin (Days 2–5), followed by RepSox (Days 5–8), but the rationale for this timing and factor selection is not well explained. It is unclear whether these cues act through their anticipated signaling pathways or how each contributes to the final phenotype. For example, it would be informative to compare iECs generated without Norrin and vitronectin but with RepSox, or vice versa. Without these controls, it is difficult to determine whether all three factors are necessary or if one alone is sufficient.

We thank the reviewer for this insightful comment. Norrin and vitronectin were included in the early differentiation phase immediately following mesodermal induction to promote retinal EC specification. Successful endothelial differentiation was confirmed on day five by robust CD31 expression. RepSox was incorporated during the later maturation phase to enhance barrier function, consistent with its reported role in supporting retinal EC maturation. In our additional experiment, iECs cultured without Norrin and vitronectin possessed minimal β -catenin activation and junctional localization and failed to show Fz4 endocytosis (New Fig. S1C and S1D). As the iECs represent a negative control for the iRECs treated with Norrin and vitronectin, these results confirm that Norrin and vitronectin induce a retinal-specific EC signature and phenotype that is absent from non-tissue specific iPSC-derived ECs.

To validate the temporal contributions of these factors, we performed RT-qPCR at day five with or without Norrin and vitronectin supplementation (New Fig. S1E). The iRECs differentiated without Norrin and vitronectin had decreased expression of the retinal EC marker, FZD4, compared to control. Their expression of the retinal EC-associated genes, CLDN10, CLDN23, CLDN2, CLDN5, and IGF2 expression was also trending toward a decrease relative to control, demonstrating the essential contributions of Norrin and vitronectin to retinal EC development and maturation. We further evaluated the role of RepSox in barrier development using TEER and permeability assays. iRECs cultured without RepSox exhibited decreased TEER and impaired barrier function over four days, whereas RepSox supplementation significantly enhanced barrier integrity and reduced permeability (New Fig. S3A-B). These results demonstrate that all three factors (Norrin, vitronectin, and RepSox) are essential to deriving mature and functional retinal-specific ECs

Fig. S1C. Expression of β -catenin and ZO-1 proteins on pre-iRECs and iECs

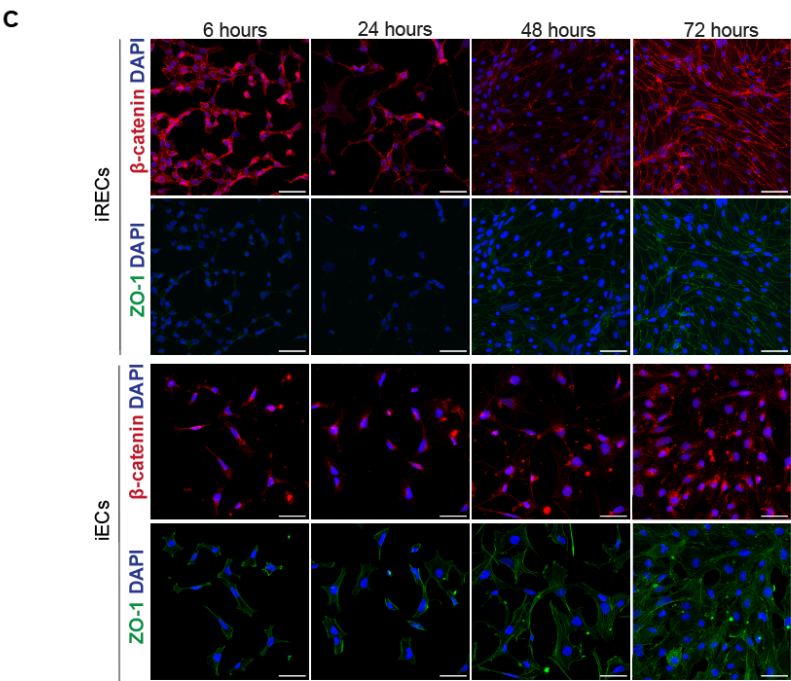


Fig. S1D. Flow cytometry analysis of iECs

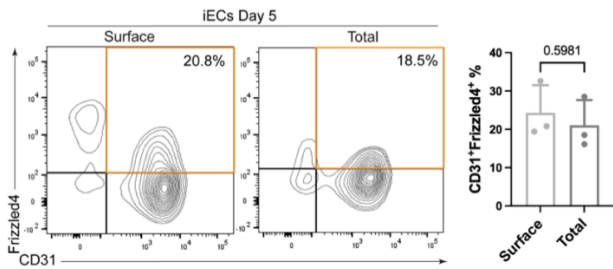


Fig. S1E qRT-PCR analysis of iRECs treated with or without Norrin/vitronectin

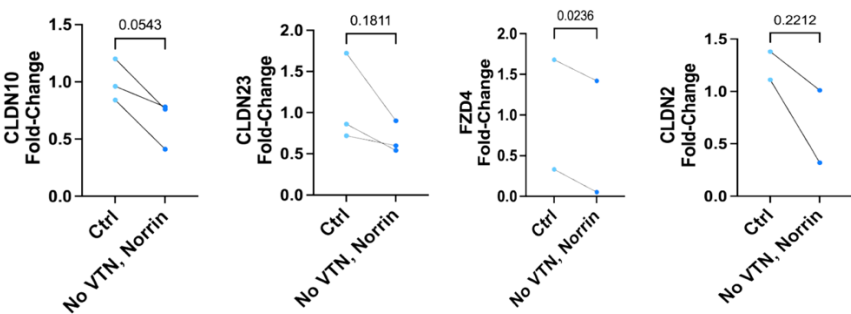
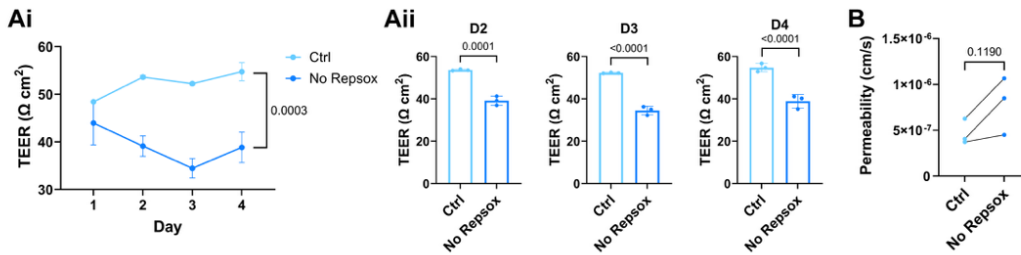
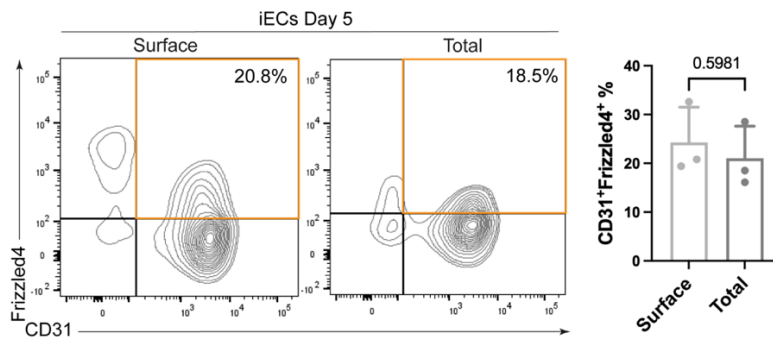


Fig. S3A-B. Barrier function measurement of iRECs treated with or without Repsox



2. The point about Frizzled-4 expression and internalization is not sufficiently clear. The authors state that ~50% of pre-iRECs undergo Fz4 internalization (Fig. 1Ciii), and that ~66% of CD31+ pre-sorted iRECs express Fz4 (Fig. 1Civ), but it is unclear whether this reflects surface or total expression. Additionally, similar surface vs. total Fz4 analysis is not provided for control iECs. In Fig. 1Cii, the addition of Norrin appears to reduce the CD31+Fz4+ surface population—possibly due to internalization—but this interpretation remains speculative. A side-by-side comparison of surface and total Fz4 in both iRECs and iECs would help clarify whether Fz4 internalization is specifically induced by Norrin signaling or reflects general heterogeneity in the differentiation process.

We thank the reviewer for bringing up this important point. The Fz4 expression shown in Fig 1Civ represents surface expression, and we have updated the figure legend and main text to clarify this. In addition, we performed flow cytometry analysis of both surface and total Fz4 expression in iECs on day 5 of the differentiation (**New Fig. S1D**). Unlike iRECs, iECs showed no significant difference between surface and total Fz4 expression, indicating that Fz4 internalization is specific to iRECs and likely reflects activation of the Norrin/Fz4 signaling pathway.



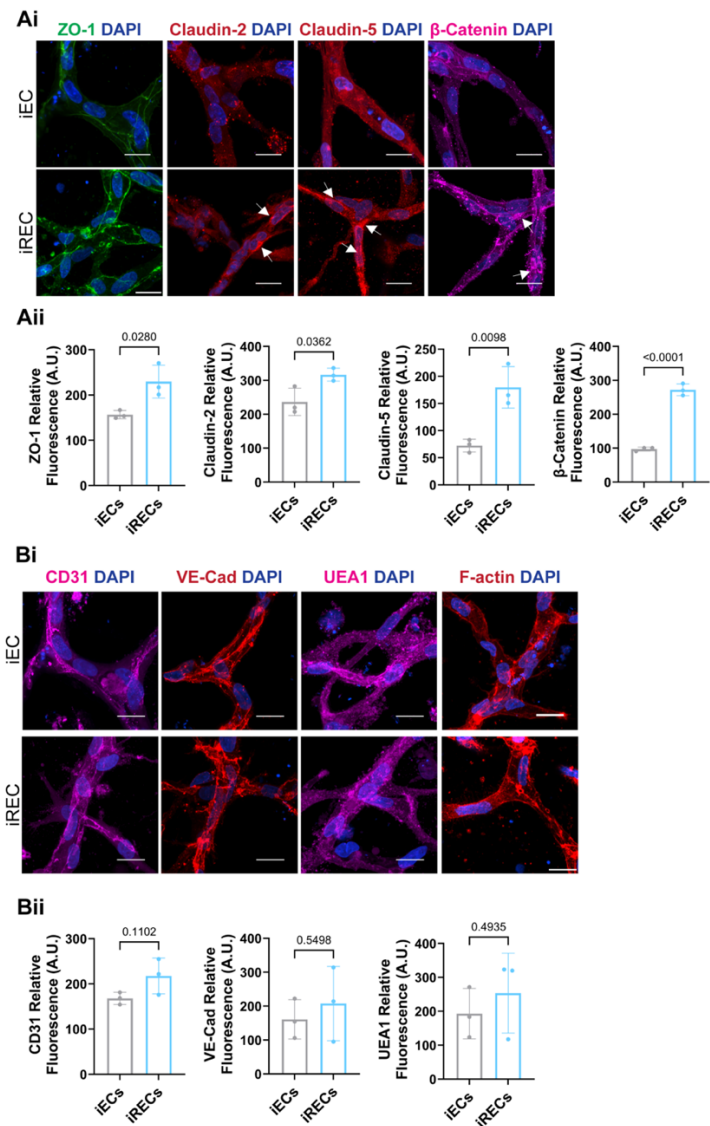
3. Overall, there is limited evidence distinguishing iRECs from control iECs. The main phenotypic differences appear to be the expression of Claudin-2 and Occludin (Fig. 1), but these observations are not supported by quantitative measurements such as western blotting, flow cytometry, or mean fluorescence intensity from immunostaining. Without such data, it is difficult to assess the robustness and extent of the reported differences. On the transcriptomic side, the authors identify several differentially expressed genes—such as FZD4, IGF2, CLDN10, CLDN23, VTN, and FN1 (Fig. 3)—but do not benchmark these against primary HRECs or retina-specific vascular datasets. As a result, it remains unclear how closely iRECs approximate native retinal endothelial cells. It would also strengthen the conclusions to examine how these gene expression patterns change when Norrin/vitronectin or RepSox are individually excluded.

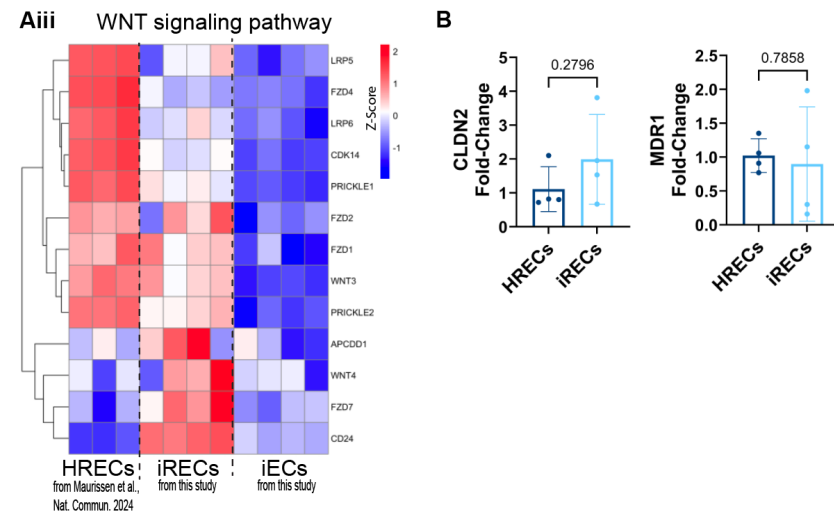
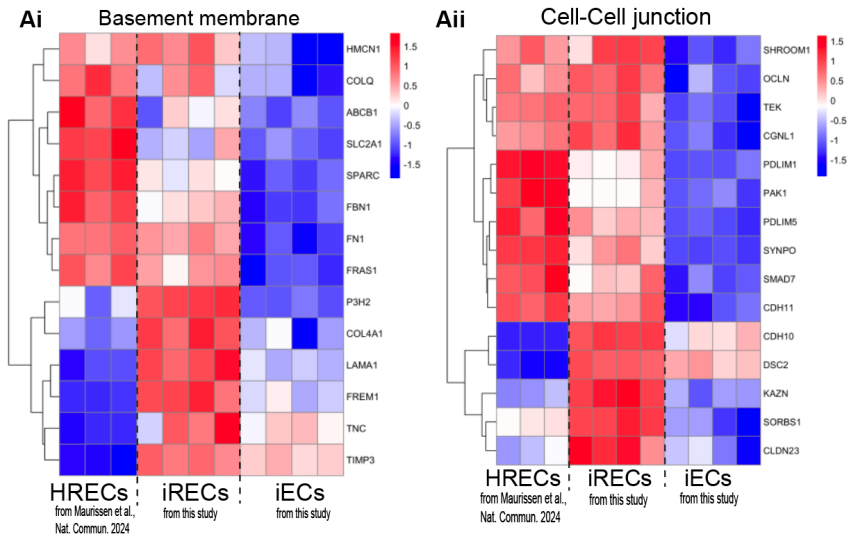
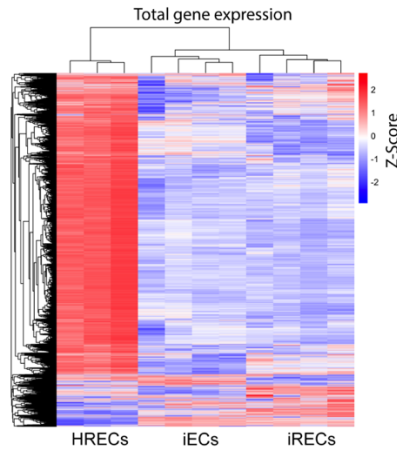
We thank the reviewer for this thoughtful and detailed comment. To strengthen our characterization of iRECs, we have now included quantitative analyses of barrier functionality and junctional and endothelial markers (New Fig. S6-9), providing functional and protein-level validation of the phenotypic differences between iRECs and iECs observed in Fig. 1. These data confirm that the enhanced expression of junctional proteins in iRECs is robust and reproducible, supporting the distinct barrier functionality and junctional phenotype conferred by retinal-specific cues.

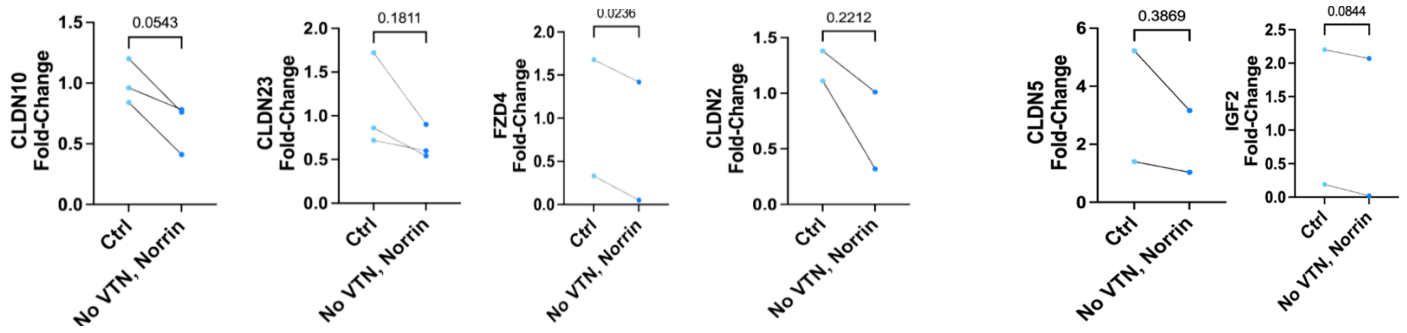
To benchmark our transcriptomic data, we compared iRECs to human primary retinal endothelial cells (HRECs) from Maurissen et al. (Nat Commun, 2024). Although global heatmap similarity was not complete, this is likely due to donor variability and culture differences, the iRECs exhibited overlapping expression across multiple retinal endothelial gene sets, underscoring their alignment with human retinal identity (New Fig. S12A). We further investigated the retinal endothelial genes CLDN2 and MDR1 by RT-qPCR and confirmed that iRECs possessed expression patterns similar to HRECs (New Fig. S12B).

Finally, to assess the contribution of factors included during differentiation, we performed RT-qPCR at day five of iREC differentiation with or without Norrin and vitronectin supplementation (New Fig. S1E). The iRECs differentiated without Norrin and vitronectin had decreased expression of the retinal EC marker, FZD4, compared to control. Their expression of the retinal EC-associated genes, CLDN10, CLDN23, CLDN2, CLDN5, and IGF2 expression was also trending toward a decrease relative to control, demonstrating that Norrin and vitronectin are critical drivers of retinal endothelial maturation.

Collectively, these results provide transcriptomic, proteomic, and functional evidence to distinguish iRECs from iECs and confirm that retina-specific signaling cues direct the emergence of tissue-relevant endothelial phenotypes.







4. There is no evaluation of DR-like responses in control iECs. The authors show that iRECs respond to high glucose and hypoxia (Fig. 2D–E), recapitulating features of diabetic retinopathy. However, without performing the same experiments in iECs, it is unclear whether these responses are specific to the iREC phenotype or simply reflect a general property of iPSC-derived endothelial cells. This comparison is essential to support the claim that iRECs provide a tissue-relevant model for retinal vascular dysfunction.

We thank the reviewer for this important comment. To directly assess whether the DR-like responses are specific to the iREC phenotype, we performed parallel experiments using iECs under identical diabetic stress conditions. iRECs exhibited a significant and progressive reduction in TEER from day 2 to day 4 compared to untreated controls (Fig. 2Diii, New Fig. S8D). Although iECs also showed a general decline in TEER under DR conditions, the changes were not statistically significant at individual time points (New Fig. S8E). Notably, the magnitude of barrier disruption was greater in iRECs than in iECs (New Fig. S8F), indicating enhanced sensitivity to DR-associated stress.

To further validate this observation, we quantified network morphology and junctional protein expression. Within 3D networks, DR-treated iRECs demonstrated marked reductions in network branch length, volume, and lumen diameter as well as ZO-1, CD31, VE-cadherin, Claudin-5, and β -catenin localization at cell–cell contacts (Fig. 2Ei-ii and New Fig. S9A), consistent with impaired barrier integrity. Initial testing with iECs in 2D demonstrates that there is no difference in ZO-1, Claudin-5, and VE-Cadherin junctional protein expression between DR and control conditions (N = 1) (New Fig. S8G, for reviewers only). Corroborating this, iEC networks treated with DR conditions possessed branch lengths, surface areas, network volumes, and lumen diameters consistent with the control groups (New Fig. S9B). Similarly, while CD31, VE-Cadherin, Claudin-5, and β -catenin protein expression are trending toward a decrease in these DR iEC networks, the reduction is not statistically significant (New Fig. S9C). Together, these findings demonstrate that iRECs exhibit better functional, structural, and molecular responses to DR stress compared to non-tissue-specific iECs, supporting their retinal identity and validating them as a tissue-relevant in vitro model for studying DR-associated vascular dysfunction.

Fig. 2Diii and Fig. S8D. TEER measurement of iRECs with or without DR treatment

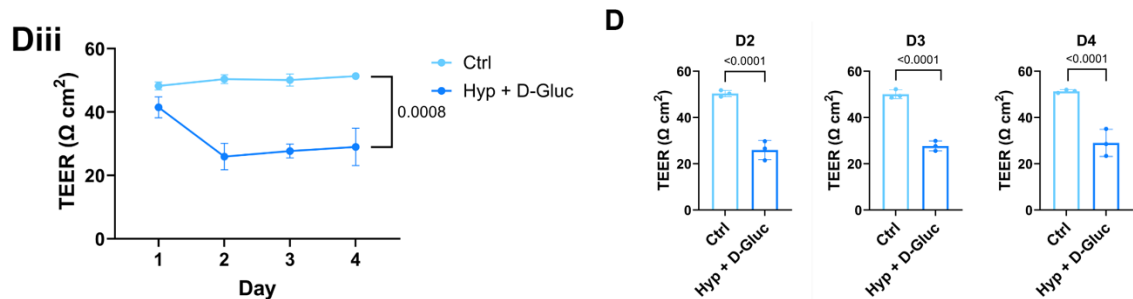


Fig. S8E. TEER measurement in iECs with or without DR treatment
Fig. S8F. Relative TEER change in iECs and iRECs after DR treatment

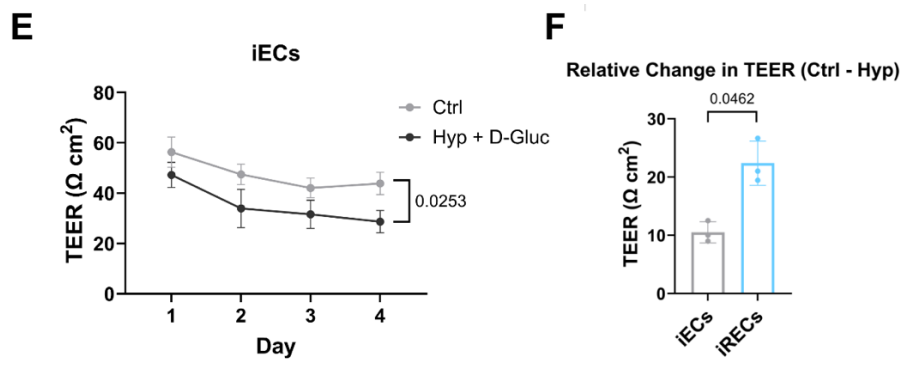


Fig. S9A. 3D iREC vascular networks with or without diabetic treatment

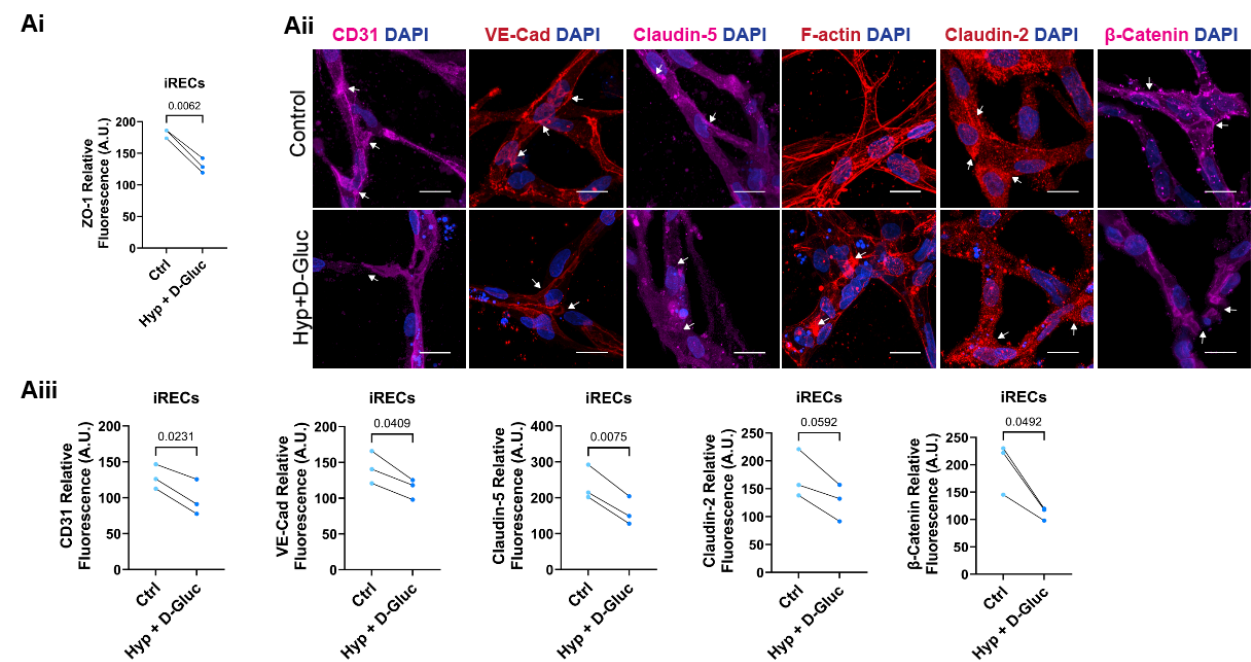


Fig.S8G. 2D iECs with or without DR treatment (Data for reviewers only)

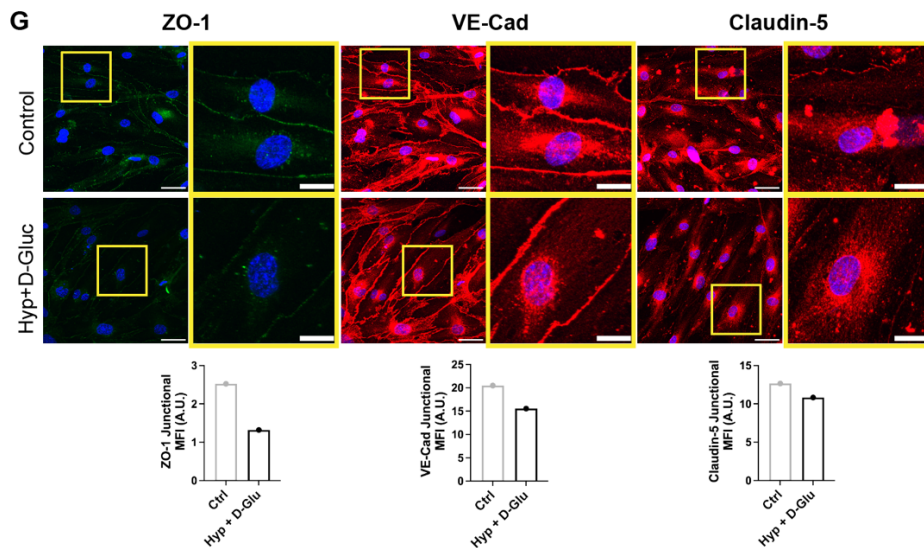


Fig.S9B. Morphology analysis of iEC vascular networks with or without DR treatment

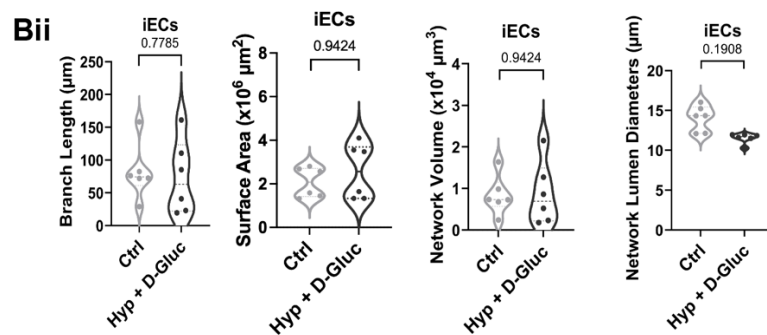
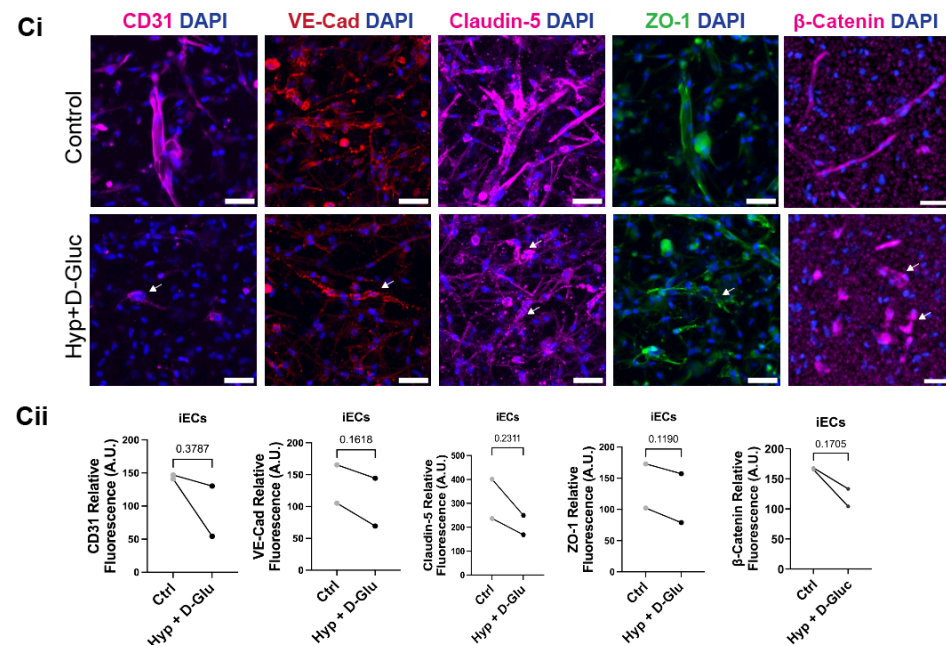


Fig.S9C. 3D iEC vascular networks with or without DR treatment

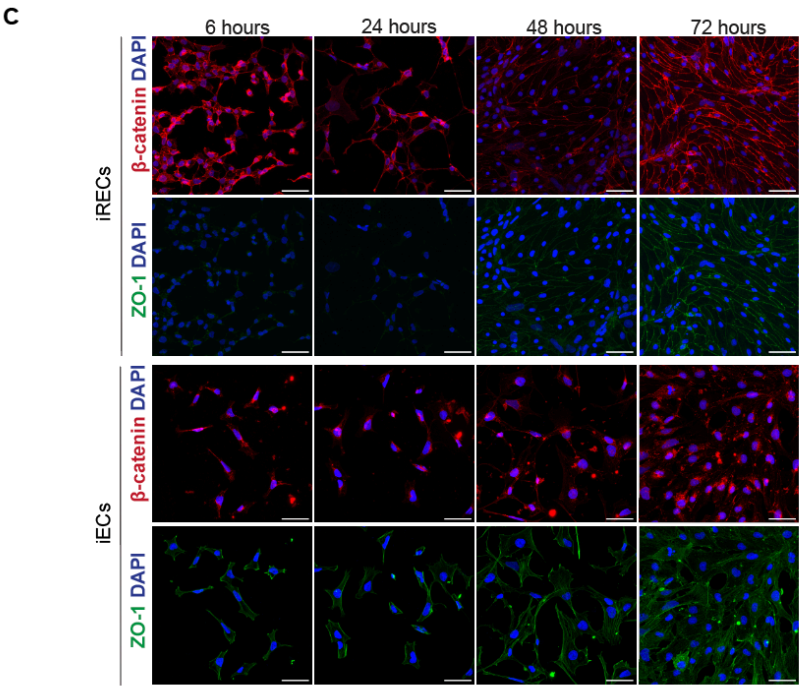


5. The transcriptomic analysis lacks tissue-specific benchmarking. While the authors show that iRECs differ from iECs and exhibit Wnt pathway enrichment (Fig. 3), they do not compare these profiles to primary human RECs or to publicly available retinal vascular datasets. As a result, the claim that iRECs “recapitulate

fundamental and developmental REC markers” feels overstated. The data suggest tissue specificity but do not confirm it. In addition, validating Wnt pathway activation at the protein level—such as through nuclear β -catenin localization—would further support the interpretation of pathway engagement.

We thank the reviewer for this thoughtful suggestion. We initially did not compare iRECs with human retinal ECs due to concerns regarding the tendency of primary cells to lose their tissue-specific phenotypic characteristic when removed from their original environment. As an alternative, we compared our samples to human primary retinal endothelial cells from Maurissen et al. (Nat Commun, 2024). The detailed results are discussed above in our response to comment 3, iRECs exhibited transcriptomic profiles similar to HRECs, including enrichment of retina-associated endothelial markers and Wnt pathway components, thereby substantiating their retinal identity.

To further support Wnt pathway activation at the protein level, we examined β -catenin localization during iREC differentiation. In pre-iRECs, β -catenin transitioned from cytoplasmic/nuclear positioning to junctional localization within 48 hours, supporting Wnt/ β -catenin signaling activation and downstream expression. This activation concided with ZO-1 expression and downregulation of ETV2, further supporting Wnt/ β -catenin pathway activation and EC maturation (New Fig. S1C). In contrast, iECs displayed minimal β -catenin activation and junctional localization. These results confirm that Norrin-induced Wnt/ β -catenin signaling is functionally engaged during iREC specification, supporting our conclusion that iRECs recapitulate key developmental and retinal endothelial features.



6. The in vivo studies omit iECs as a therapeutic control. In the OIR model, iRECs reduce vaso-obliteration and neovascularization compared to PBS-treated eyes (Fig. 4), but without a direct comparison to iECs, it is not possible to determine whether the observed benefit is specific to the iREC phenotype. Including iECs as a control would have clarified whether iRECs offer superior or tissue-specific therapeutic potential. It would also have been informative to assess in vivo phenotypic maturation of the implanted cells using human-specific markers or transcriptomic analysis, to determine whether iRECs acquire retina-specific signatures after engraftment.

We thank the reviewer for this insightful comment. To address this, we performed additional in vivo experiments including iECs as a control to directly compare their therapeutic efficacy with iRECs. Consistent with our previous findings, iEC treatment reduced both neovascularization (NV) and vaso-obliteration (VO) in OIR retinas (New Fig. S14A). Using human-specific UEA1 and CD31 staining, both iECs and iRECs were detected in close association with host vasculature and were associated with reduced vascular leakage (New Fig.4C, S14B, 4G and S14F). Moreover, rhodamine–dextran perfusion confirmed that CD31 expressing iREC-

derived lumen-like structures were functionally perfused, indicating successful vascular integration and maturation (New Fig. 4H). Notably, iRECs demonstrated better efficacy than iECs, restoring physiological vascular network with smaller segment volumes and lumen diameters while iECs did not impact these parameters (Fig. 4E and New Fig. S14D). These results emphasize that iRECs possess enhanced retinal-specific reparative potential compared to non-tissue specific iECs.

Fig. S14A-B. iECs injected OIR retina

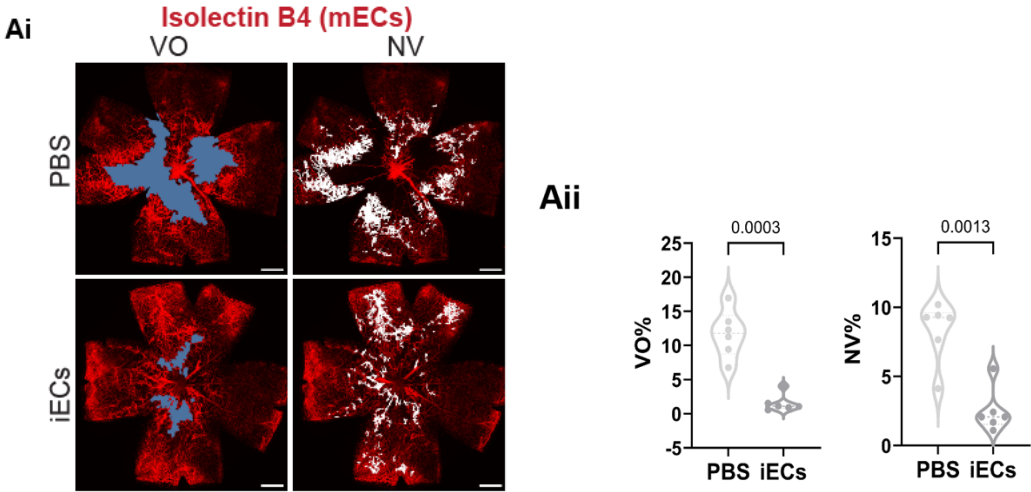


Fig 4E. Quantification of mouse vascular network segment volume and lumen diameter across PBS and iREC injected groups

Fig. 4C. iREC injected OIR retina interacting with host vasculature

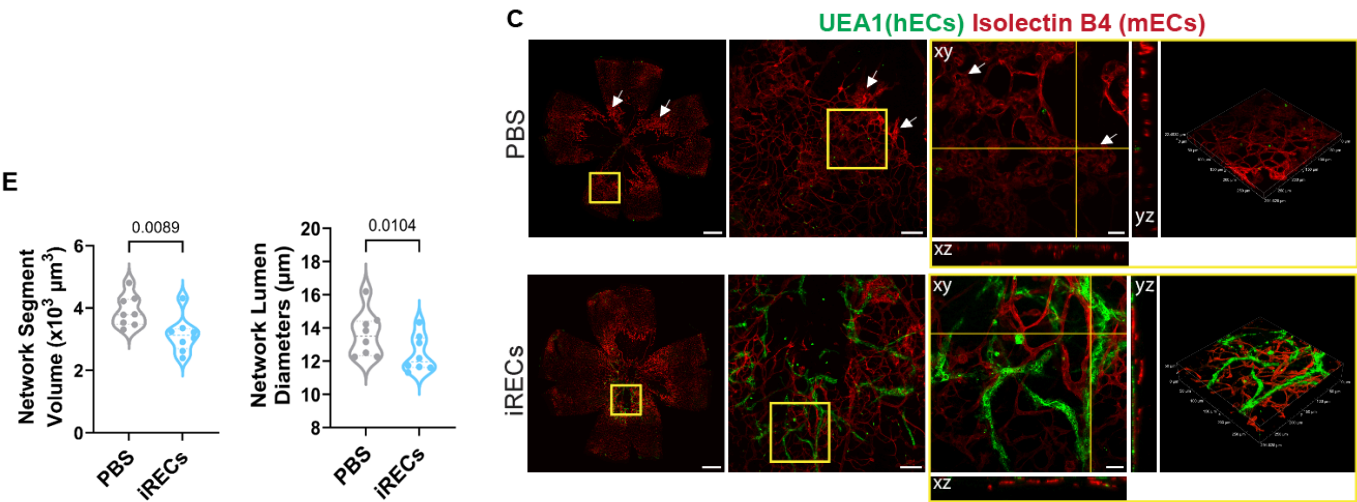


Fig. S14B. iECs injected OIR retina

Fig. S14F. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iEC injected OIR retinas

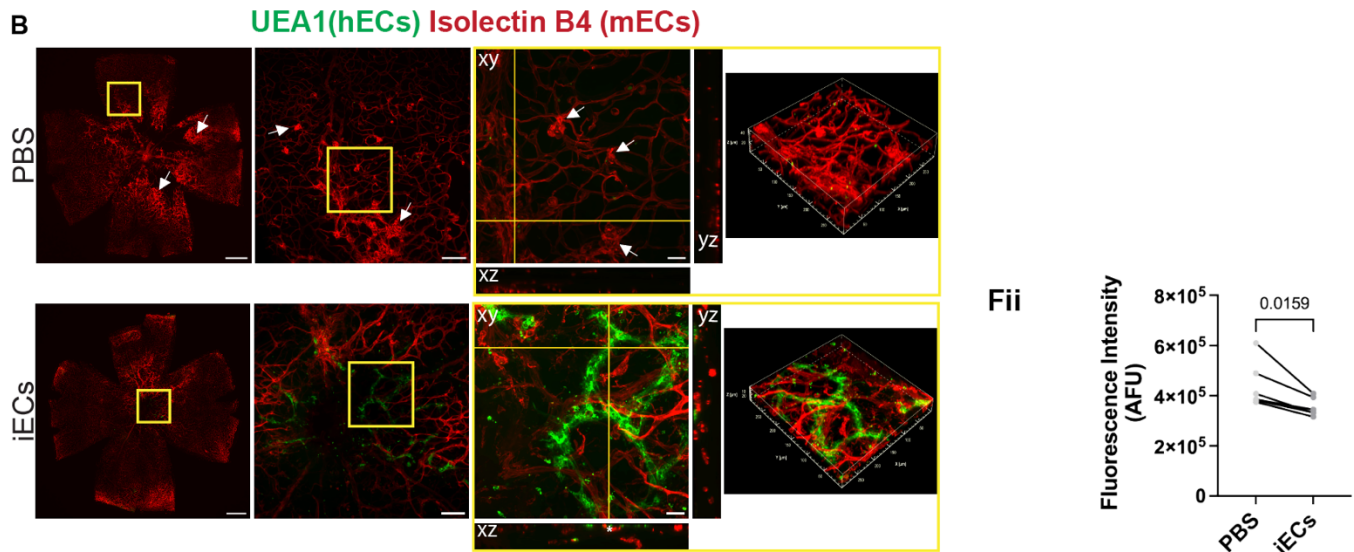
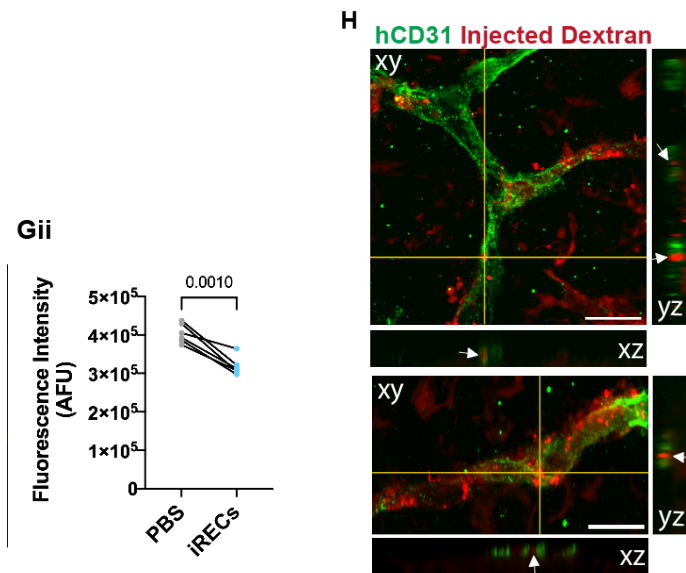


Fig.4Gii. Quantification of fluorescence intensity of 70 kDa rhodamine dextran extracted from iREC injected OIR retinas.

Fig. 4H. iREC networks perfused by injected 70 kDa rhodamine dextran within OIR retinas



7. The microfluidic iBRB model lacks appropriate control comparisons. The authors present a perfusable iBRB-on-chip system using iRECs (Fig. 5), but without including iECs as a control, it is difficult to assess whether iRECs confer any specific functional or structural advantage. In addition, previous studies have shown that iPSC-derived ECs often require stromal or mural support to form stable vascular networks. It is unclear whether the iREC networks shown in Fig. 5A–D were formed in isolation or with supporting cells—this should be clarified.

We thank the reviewer for this valuable comment. The vascular networks shown in Fig. 5A–D were formed using iRECs alone to directly evaluate their intrinsic capacity to form perfusable vasculature relative to iECs. Although iREC and iEC network size and morphology were comparable, iRECs exhibited lower permeability and thus increased barrier properties relative to iECs, (New Fig. 5E, S15B and S15Ciii). This demonstrates that the iRECs can generate a functional and physiologically accurate iBRB, displaying their

biological fidelity and advantage over non-tissue specific ECs for iBRB modeling. When iRPCs were included during network formation, iRECs developed smaller, capillary-scale networks with further decreased permeability (New Fig. 5I-J), closely resembling human retinal microvasculature¹⁻⁵. These results highlight the retina-specific functional advantage of iRECs and the supportive role of iRPCs in establishing physiologically relevant iBRB architecture and barrier properties.

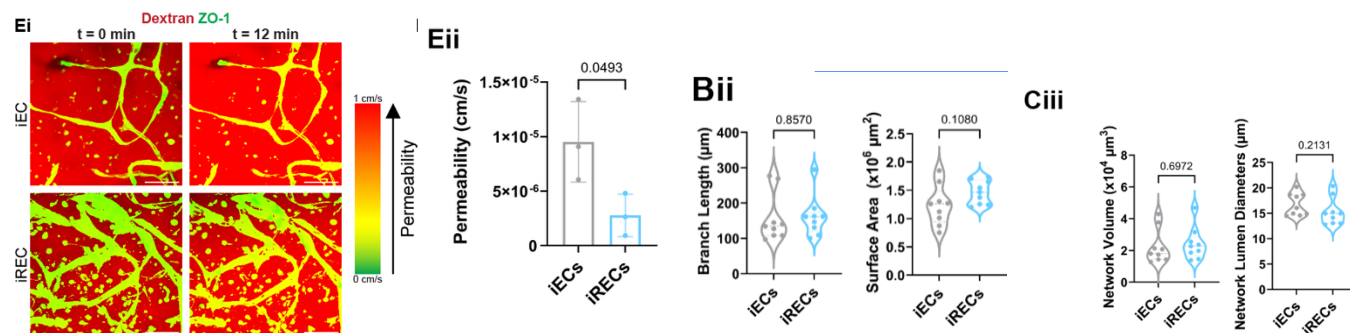


Fig. 5I. iRECs-iRPCs co-cultured MPS

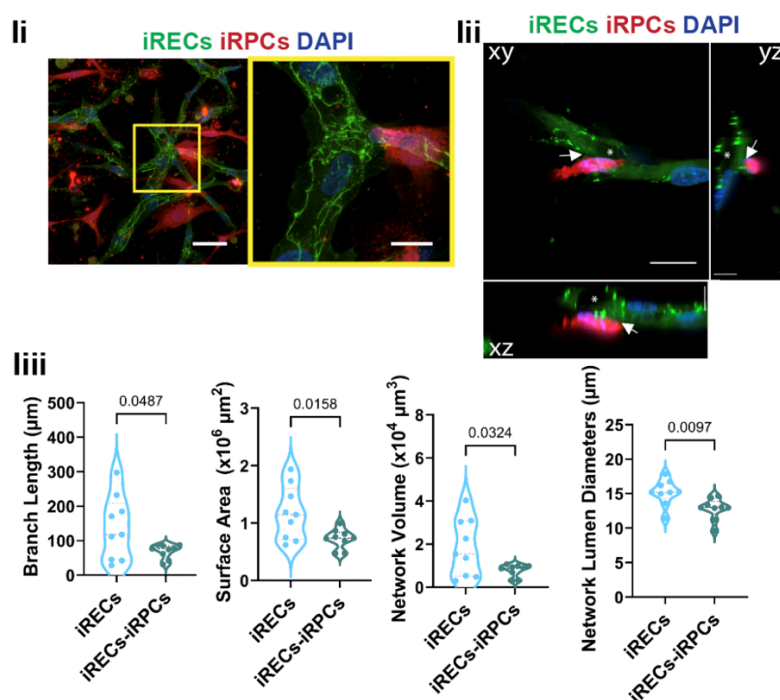
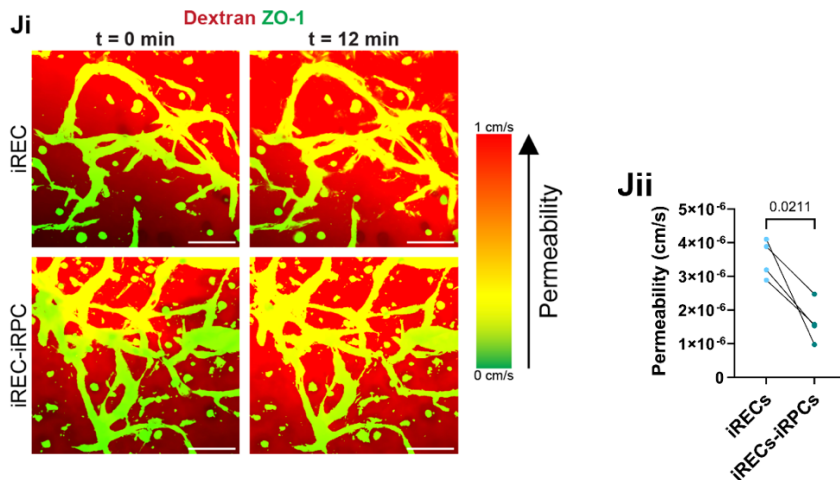


Fig. 5J. Permeability assay of iRECs-iRPCs co-cultured MPS



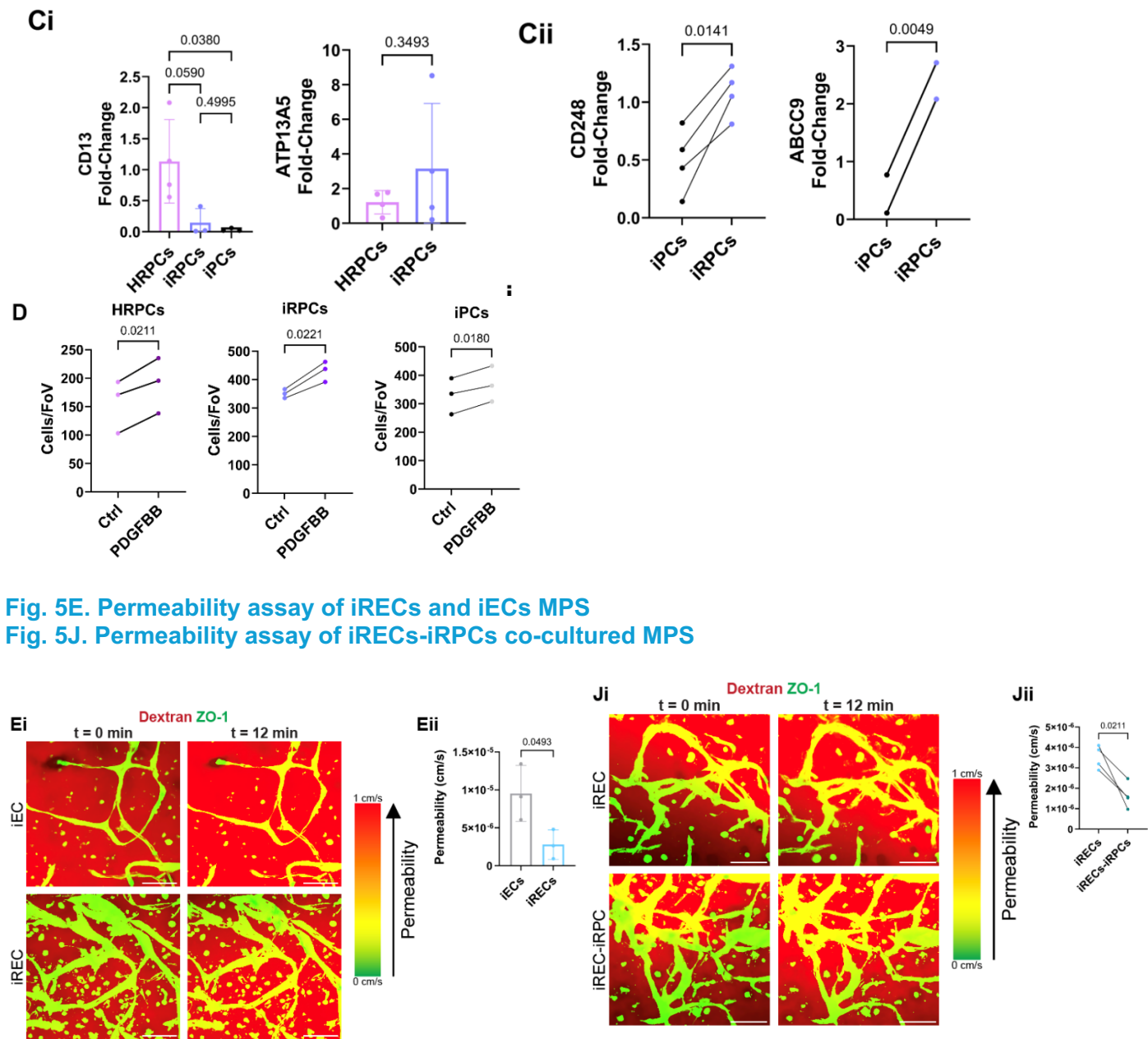
8. The characterization of iPSC-derived retinal pericytes (iRPCs) is underdeveloped. Their retinal specificity is primarily inferred from Fz4/PDGFR β co-expression (Fig. 5F–G), but this conclusion appears premature. There is no comparison to control iPSC-derived pericytes or other mesenchymal populations, and no functional assays are provided to assess pericyte competence. Moreover, the protocol for iRPC generation is introduced with minimal validation, and the role of Norrin in their specification is not explored in depth. Also, the conclusion that the iREC–iRPC co-culture “recapitulates tissue-specific retinal-like vascular networks” is not adequately supported. While the structural organization is evident, the study does not show that this model performs better than alternative combinations, such as iECs with non-retinal pericytes. The authors could reasonably state that the system recapitulates physiological endothelial–pericyte interactions, but the tissue-specific aspect of the model is not convincingly demonstrated.

We thank the reviewer for this thoughtful comment. The characterization of retinal pericytes indeed remains limited in the literature. To address this, we utilized RT–qPCR analyses to assess the retinal specificity of iRPCs at the transcriptomic level. The iRPCs expressed comparable levels of the pan-pericyte marker CD13 to HRPCs and iPCs (New Fig. S16Ci), and similar levels of the retinal-specific marker ATP13A5 to HRPCs (New Fig. S16Ci). Additionally, they exhibited elevated expression of the pericyte-associated genes CD248 and ABCC9 relative to iPCs (New Fig. S16Cii).

To investigate functionality, we performed a Transwell migration assay in the presence and absence of platelet-derived growth factor BB (PDGFBB), an EC secreted factor critical to iBRB development and thus a stimulus the iRPCs must respond to^{8–11}. The HRPCs, iRPCs, and iPCs all exhibited an increased migration to the basolateral side of the Transwell in the PDGFBB group compared to the control (New Fig. S16D), confirming that iRPCs retain key pericyte function.

Morphological and functional analyses in the MPS revealed that iREC–iRPC co-culture interactions contributed to a decrease in vascular network branch length, surface area, volume, and lumen diameter relative to the iREC only group (New Fig. 5liii and S16F), recapitulating iBRB network morphology and the luminal size of human iBRB capillaries^{1–5}. The iRPCs also increased the barrier properties of the iREC networks, decreasing their 70 kDa rhodamine dextran permeability to a level consistent with in vivo permeability¹² (New Fig. 5J). Taken together, this demonstrates the retinal-specific signature and biological fidelity of the iRPCs and their ability to develop morphologically and functionally relevant iBRB networks.

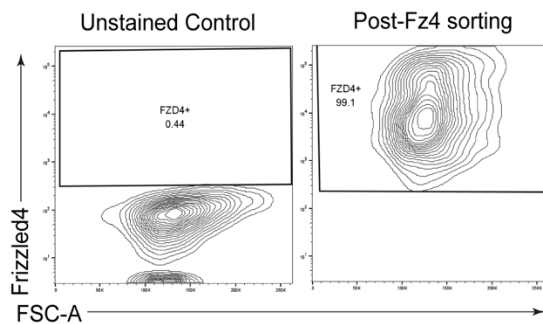
We acknowledge that additional characterization would further strengthen the study and have noted this as a limitation in the revised “*Limitations of the study*” section.



Minor Concerns

In Fig. 1, it would be helpful to report the efficiency of the Fz4+ sorting step. Providing post-sort purity (e.g., via flow cytometry) would help assess the robustness of the protocol and the homogeneity of the iREC population.

We thank the reviewer for bringing up this important point. To address this, we performed flow cytometry using labeling check reagent APC (Miltrenyi Biotech, #130-122-228) to verify the purity of the cells after the Fz4 sorting step. We found that 99.1% of the cells were Fz4-positive post-sort, confirming the robustness of the protocol and the homogeneity of the iREC population. (Data for reviewers only)



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Response to reviewers

General comments:

To increase the rigor and confirm the reproducibility and accuracy of our work, we have updated our RT-qPCR data for Fig. S1E, network analysis for Fig. S9Bii, and fluorescence intensity data for Fig. S8D and S9Cii to include additional biological replicates. We added a ZO-1 stain to Fig. S2D to clearly delineate cell-cell borders to confirm Dil-LDL uptake. We also provided more accurate representative images for Fig. S9Bi and Ci and changed the color scheme in Fig. S16 Eiii-F to ensure consistency with Fig. 5li-ii and S16Ei-ii.

Reviewer comments:

Reviewer #1:

The authors have conducted key additional experiments and provided new data supporting the superior performance of iRECs (compared to generic iECs) in in vitro disease modeling. Several concerns raised in the previous review have been addressed. However, although the in vitro benefits of iRECs are now well demonstrated, their claimed superiority in vivo remains unsubstantiated. A number of important issues persist:

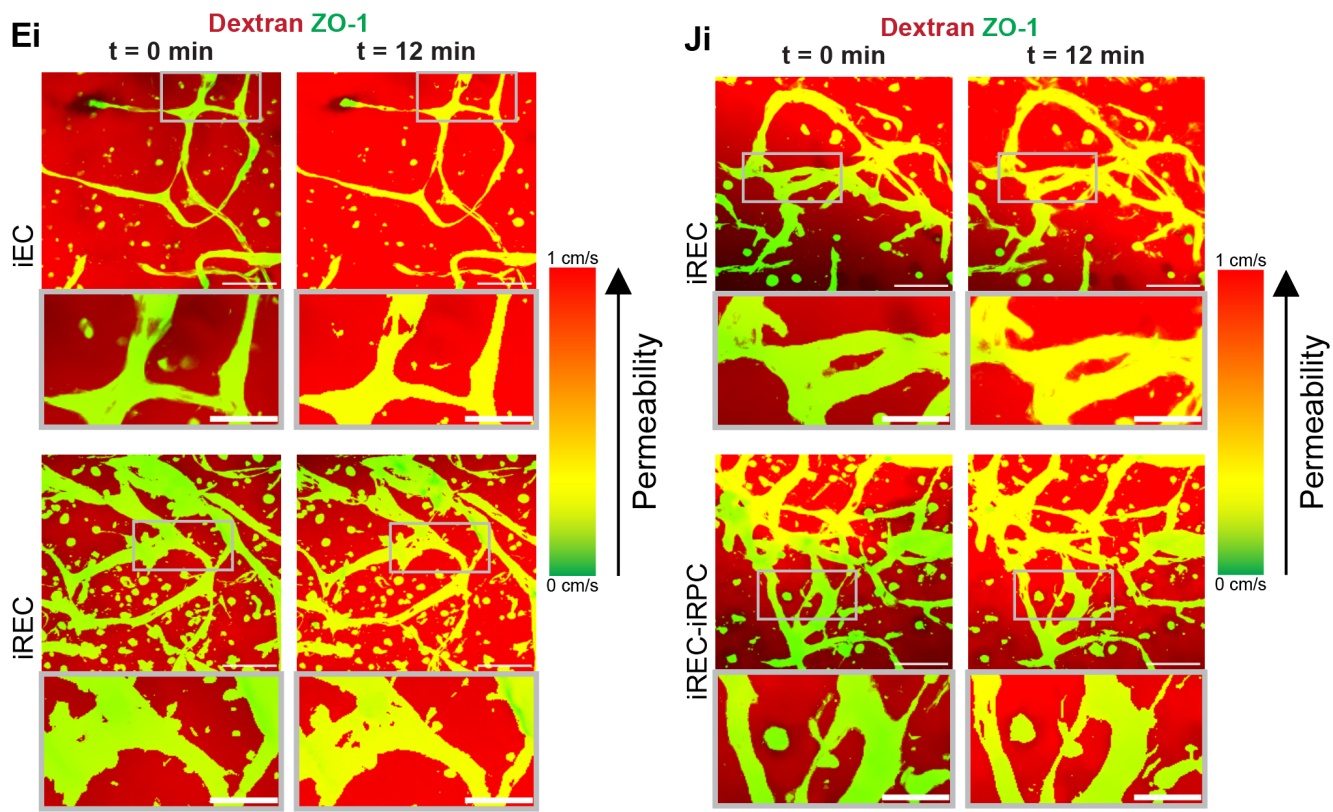
1. Vessel permeability assessment in the MPS

The method used to assess vessel permeability is unclear and requires further clarification. The authors state that they filled the hydrogel with fluorescent tracer and quantified fluorescence intensity changes within the vascular network, which, if I interpreted correctly, reflects reverse permeability. If this is indeed the intention, representative images showing differences within the vasculature should be provided, as the current images illustrate only the interstitium. Even if images reveal differences, the relevance of this assay to physiological permeability remains questionable and should be justified.

We thank the reviewer for this important question. Your interpretation is correct, we flooded the collagen-I hydrogel with 70 kDa rhodamine dextran, enabling us to quantify the permeability into the networks. To do this, we followed a standard and exhaustively validated protocol that quantifies the change in network fluorescence to establish permeability¹. As a modification to this protocol, we calculated the fluorescence intensity difference within the networks over time rather than the matrix as the flooding of the hydrogel with dextran prior to imaging was used to investigate the permeability into the networks rather than out of the networks. As the protocol has been validated for quantifying fluorescence intensity for permeability regardless of the direction of dextran flow, the use of the equation for permeability into the network is accurate and thus physiologically relevant. Previous studies have also demonstrated that the permeability into and out of cells and networks can be calculated and the permeability in either direction is indistinguishable²⁻⁵, further supporting the physiological relevance of our permeability assays and calculations.

Within the representative maximum intensity projection immunofluorescence images from Fig. 5Ei and Ji, ZO-1-GFP denotes iEC or iREC networks. As a result, the change in green network color across timepoints $t = 0$ min and $t = 12$ min, where the green networks become more yellow/red, is a result of the rhodamine dextran accumulation within the networks and thus represents network permeability into the networks rather than just displaying interstitium. We have updated the figure legend to clarify this. We also updated Fig. 5Ei and Ji to include inset images to enable an easier visualization of the network color change between $t = 0$ min and $t = 12$ min.

We added this clarification to the figure legend.



2. Discrepancies between Fig. S15 Ei/Eiii and Fig. 5Ei/Ji

Figures S15 Ei and Eiii look markedly different from Fig. 5Ei and Ji. The authors should explain this discrepancy. Additionally, why was the perfusion method, which is standard in the field not used to assess permeability directly?

Fig. 5Ei and Ji are representative real-time images that were taken during the permeability assay at $t = 0$ min and $t = 12$ min without fixing and washing. Prior to imaging, we delivered $20 \mu\text{L}$ of transport buffer to one media channel and $70 \mu\text{L}$ of TB buffer supplemented with $100 \mu\text{g/mL}$ 70 kDa rhodamine dextran to the other media channel of a device. This generated an interstitial flow that enabled the rhodamine dextran to diffuse throughout and fill the collagen type-I hydrogel. Once dextran was homogeneously distributed across the hydrogel, we began imaging the samples with the first image stack representing t_0 ($t = 0$ min). Image stacks at the same location as t_0 were taken 12 minutes later to complete the permeability assay. As rhodamine dextran was present throughout the hydrogel for both image timepoints, the images possessed rhodamine dextran signal surrounding and within ZO-1 networks.

In contrast, to visualize vascular perfusion in Fig. S15E, devices were washed with PBS for 5 minutes on a rocker to remove excess dextran within the hydrogel. Devices were then fixed in 4% formaldehyde for 20 minutes followed by 3 PBS washes. These wash steps removed the perivascular rhodamine dextran within the hydrogel without washing out the dextran located in the networks, resulting in excessive perivascular rhodamine dextran being absent from the images in the Fig. S15E. This generates the distinct images from Fig. S15E without perivascular rhodamine dextran relative to Fig. 5Ei and Ji. As perfusion imaging was performed after fixing and washing the cells, permeability measurements could not be calculated.

To ensure the difference between the images and the assays are clear, we updated the permeability and vascular perfusion descriptions within the *iBRB-on-a-chip permeability assay* section in the paper.

The permeability assay we used was designed to address the challenge associated with using collagen-I hydrogels. We encapsulate iRECs and iECs within collagen-I hydrogels to generate the MPS as collagen-I is a naturally occurring ECM component within the retina. This represents a critical advantage of our system and its ability to recapitulate the physiological environment compared to the commonly used fibrin-based hydrogels,

which lack the native retinal ECM component⁶⁻¹⁴. The “standard” perfusion method the reviewer is referring to is used for 3D models made with fibrin hydrogels. However, collagen-I and fibrin hydrogels are composed of different materials and thus they possess different mechanical and structural properties, with collagen-I hydrogels possessing greater susceptibility to contractility. While we initially tried to perfuse the networks with 70 kDa rhodamine dextran using the luminal perfusion method, we were unable to perfuse them without using bulk hydrogel perfusion due to the mechanical and contractile properties of collagen-I hydrogels. As a result, we could not accurately calculate fluorescence change outside of the networks and thus network permeability using the luminal perfusion approach. To address this, we use the well-established approach to determine permeability¹ and flood the hydrogels with 70 kDa rhodamine dextran to quantify dextran permeability from the surrounding hydrogel into the networks.

3. Gene expression comparisons between HRECs, iECs, and iRECs

The gene expression analysis shows that iECs and iRECs are far more similar to each other than either is to HRECs. This is concerning, as many genes exhibit an inverse expression pattern relative to HRECs. The authors should address this more thoroughly, discussing implications for the retinal identity and fidelity of iRECs.

We thank the reviewer for raising this important point regarding the transcriptional similarity between iECs and iRECs relative HRECs and its implications for retinal EC identity. RNA-Seq data for iECs and iRECs were generated in parallel by the same vendor using identical library preparation and sequencing pipelines. In contrast, the HREC RNA-Seq dataset was obtained from a published study¹³. Although DESeq2 normalization and VST were used to reduce technical variance within datasets, direct cross-study comparisons remain susceptible to batch effects and bias associated with the sequencing system utilized, which contribute to the apparent difference in global gene expression patterns. To highlight this limitation, we stated that “An additional challenge comes with comparing published primary HREC and iPSC-derived brain EC RNA-Seq datasets with our datasets as there is a potential for batch effects and bias associated with the sequencing system utilized” within the manuscript’s *Limitations of the study* section.

Despite these limitations, iRECs exhibit selective transcriptional similarity with HRECs in functionally relevant pathways critical to retinal-EC identity, including basement membrane, cell-cell junction, and Wnt signaling related genes. On the contrary, this similarity is not observed to the same extent in iECs. To this end, while iRECs do not recapitulate the complete transcriptional profile of HRECs, they express genes fundamental to the retinal signature of HRECs to a greater degree than iECs, supporting their retinal identity and genomic fidelity. Although iECs and iRECs share broad transcriptional features likely due to their common iPSC-derived origin, the retinal-specific expression patterns present in iRECs are limited or absent in iECs, corroborating the physiological relevance and accuracy of iRECs.

To clarify these points and strengthen the interpretation, we revised the *Discussion* section to emphasize the pathway-specific evidence underlying iREC identity.

4. Claims regarding “enhanced retina-specific reparative potential of iRECs”

The data do not sufficiently support this conclusion.

- In Fig. S14, the iEC networks (panel Fi) appear far more robust than either PBS or iREC conditions (panel E). Based on visual assessment and quantitative parameters, there are very few differences between iECs and iRECs in reparative potential.
- The two experimental batches differ substantially.
 - o Batch 1 (original submission): PBS vs iREC, with VO = 20% in PBS controls.
 - o Batch 2 (revision): PBS vs iEC, with VO = 10% in PBS controls.

This suggests a substantially milder insult in Batch 2, which may mask differences between PBS and iECs. Importantly, variability in PBS controls is 2× between batches, whereas variability in treated groups (iEC/iREC) is lower, indicating high model variability overall.

- Only two parameters showed significant differences for iRECs: network segment volume (noting that iECs were nearly significant, $p = 0.059$ —was power calculated?) and vessel diameter, whose relevance to regeneration is unclear.

- VO, NV, and Dextran fluorescence intensity differed significantly for both iECs and iRECs, while branch length and surface area did not differ for either group. This pattern suggests more similarities than differences between iECs and iRECs in vivo.

Taken together, these findings may reflect assay variability rather than a true enhanced reparative potential of iRECs in comparison to iECs. While the authors were overall cautious in their claims, stating mostly that iRECs have a regenerative ability, the authors are encouraged to either provide additional evidence through a direct head-to-head comparison of iECs and iRECs within the same experimental batch, or remove claims of in vivo superiority (page 34, last sentence) and include this as a limitation of the study.

We thank the reviewer for these important and helpful points. While the OIR mouse model is the standard and most widely used model for studying DR angiogenesis/neovascularization and the discovery and testing of therapeutics^{15,16}, there is inevitable variability within and across batches. This is due to differences in pup-to-pup handling, weight, nursing, light exposure, etc., which can not be completely accounted for and mitigated. While a direct comparison between iECs and iRECs within the same mouse would provide the best mechanism to evaluate their therapeutic and reparative capabilities, we are unable to perform this direct iEC and iREC comparison as there is a need for a PBS control in the contralateral eye to confirm initial injury. A bilateral transfer of biological material across eyes has also been documented¹⁷, further preventing a reliable direct comparison of iRECs and iECs within the same mouse due to the potential of cell migration and paracrine signaling confounding results and hindering data analysis and statistical comparisons. We have removed the claim that iRECs have enhanced capabilities relative to iECs and have updated the *Limitations of the study* section.

5. Concerns with perfusion shown in Fig. 4H

The perfusion data are not convincing. There appears to be as much (or more) red signal outside the vessels as inside. The small region where red overlaps with green seems localized and potentially artifactual. If the vessels are leaky—making perfusion difficult to assess—this should be explicitly addressed. The authors should clarify:

- Why such extensive extravascular red signal is present,
- Whether the chosen time point is appropriate, and
- How leakage affects interpretation of perfusion success.

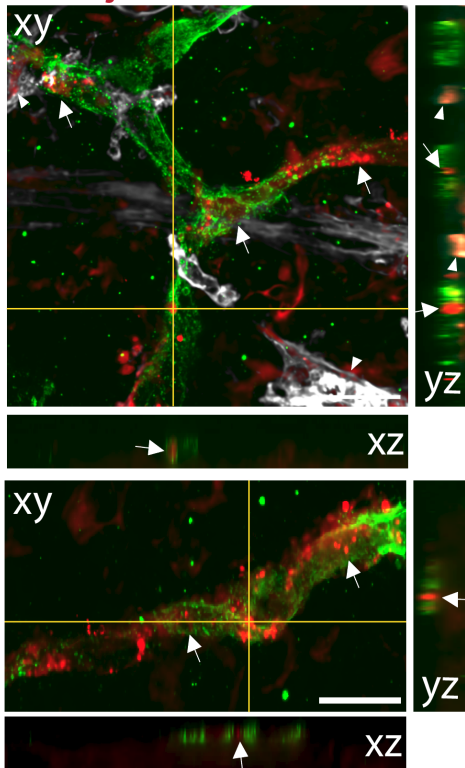
We thank the reviewer for this insightful question. Fig. 4H shows a representative retinal region containing iREC-derived vascular networks after iREC injection. This experiment was designed to determine 1) if iREC networks are functionally connected to the host vasculature and 2) whether iREC treatment reduces retinal vascular permeability compared with PBS controls. The presence of rhodamine dextran within the lumina of iREC networks demonstrates that these structures are perfusable and connected to the host vasculature. The extravascular rhodamine dextran signal is associated with the native mouse vasculature and iREC-derived vessels and is expected under the experimental conditions. In the original figure, we focused on highlighting iREC network perfusion and therefore did not include the marker for host vasculature. To address this, we updated the figure to include the Isolectin B4 stain for mouse ECs. Although there is some residual background rhodamine dextran signal present, this is expected since we performed the permeability assay on P17, when vaso-obliteration and neovascularization are at their peak in the model. This timepoint was chosen as it is the standard in the field for therapeutic evaluation, including permeability assays¹⁸⁻²¹. This timepoint also corresponds to the most extreme case of retinal injury, allowing us to evaluate the full reparative capacity of the iRECs. Accordingly, increased vascular permeability is anticipated, and a fully sealed EC barrier is not expected in either host or iREC-derived vessels at this time point.

Retinal vascular permeability was quantified by extracting extravascular 70 kDa rhodamine dextran present within the entire retina using N,N-Dimethylformamide and measuring fluorescence intensity. As retinal vascular permeability quantification is calculated based on the amount of extravascular rhodamine dextran within a retina, the leakage within each retina is fundamental to establishing its permeability. The less leakage present, the lower the permeability calculated, and thus the greater the vascular repair following iREC treatment. For perfusion of iREC networks, we considered any iREC network with rhodamine dextran encapsulated within its lumina perfusable.

We have revised the text and Fig. 4 to address and clarify the perfusion and permeability assay and outcomes.

H

CD31 (hECs) Isolectin B4 (mECs)
Injected Dextran



General Comments

- Many graphs use line plots to compare discrete conditions (e.g., PBS-iPECs, PBS-iECs, Ctrl-PDGFBB, iPCs-iRPCs). This is inappropriate, as line plots imply continuous or paired data (e.g., before-after). This raises concern about incorrect statistical treatment, particularly since figure legends do not specify the statistical tests used. If the conditions were indeed paired, that should be explicitly clarified—but based on the experimental design, this seems unlikely.

We thank the reviewer for raising this concern. As clarification, all line plots or before-after plots correspond to data that we performed two-tailed paired Student t-tests on as they all represent paired data. All assays where cells from the same biological replicate were exposed to different conditions in parallel underwent paired testing. The *in vivo* assays were also analyzed utilizing two-tailed paired Student t-tests as each individual mouse was injected with PBS in one eye and iRECs or iECs in the contralateral eye. Paired testing was utilized to account for batch effect and sample bias.

We included an explanation of which data underwent paired analyses within the *Statistics* section of the paper where we stated “Two-tailed paired Student t-tests were performed to statistically compare all GLUT1 uptake and diabetic retinopathy assay data. All before-after plots resemble paired data and denote two-tailed paired Student t-test analyses”. To clarify this section and avoid confusion, we have updated this description to include line plots and *in vivo* assays.

- All statistical methods and sample sizes (n) should be included in each figure legend to allow proper evaluation of the results.

We thank the reviewer for highlighting this important point. The statistical methods and sample sizes utilized within each figure are included within the *Statistics* section of the manuscript (see below). If the sample size for a given figure/result is not N = 3, n = 3, this is specified within the figure legend.

Statistics:

Biological replicates (indicated as N) are defined as separate differentiation replicates and technical replicates (indicated as n) come from the same N cultured in different tissue culture wells. All *in vitro* quantifications are N = 3 with n = 3 unless otherwise indicated in the figure legends. For *in vivo* experiments, mice number in each experiment is indicated as n. All bar graphs represent means $\pm$ SD. Graphpad Prism (v10.2.3.) and JMP 17 were utilized for statistical analysis. Two-tailed paired Student t-tests were performed to statistically compare all GLUT1 uptake, diabetic retinopathy, and *in vivo* assay data. All before-after plots, also known as line plots, resemble paired data and denote two-tailed paired Student t-test analyses. The p-gp analyses were performed utilizing two-tailed one sample Student t-tests. For TEER assays, we utilized two-factor repeated measures ANOVA to compare cell types. Post-hoc Tukey's tests were then performed to analyze individual days. Two-tailed unpaired Student t-tests were performed as appropriate for all other statistical comparisons. Significance levels were set at $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $****p \leq 0.0001$.

Reviewer #2:

The authors have followed our suggestions.

We thank the reviewer for taking the time to review all of our edits and updates and for their helpful suggestions and feedback during the review process.

Reviewer #3:

The authors have responded thoroughly to the prior critiques, and the manuscript is clearly improved. The added controls, functional comparisons, and clarification of claims strengthen the overall rigor. The conclusions are now more measured, and the key outcomes are supported by the data. I am satisfied with the revisions. One small point that could be acknowledged in the Discussion is that, at a global transcriptomic level, iRECs remain closer to iECs than to primary HRECs, which is expected at this stage. This does not detract from the significance of the work but would help to frame the findings realistically.

We thank the reviewer for thoroughly reviewing all of our edits and updates and for their beneficial suggestions and feedback during the review process. To clarify the transcriptomic analysis and properly frame the findings, we have updated the *Discussion* section to acknowledge that while iRECs do not completely reproduce the entire transcriptional profile of HRECs, they demonstrate targeted alignment in key retinal pathways, including those related to the basement membrane, cell-cell junctions, and Wnt signaling genes. This indicates that iRECs possess a physiologically relevant retinal signature. Although iRECs and iECs share many transcriptional characteristics owing to their shared iPSC-derived origin, iECs lack the extent of retinal-specific gene expression alignment with HRECs that iRECs exhibit. These findings support the retinal identity and genomic integrity of iRECs.

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