Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In general terms the paper by Yang et al proposes the finding which is novel that the RNA binding protein Quaking 7 is up-regulated within the diabetic conditions and when up-regulated by a likely splicing factor impairs endothelial cell function in a manner that is consistent with the mechanism of diabetes induced vascular injury. I suggest the authors consider the following suggesting/questions/areas for clarification.

1) Is the increase in Quaking 7 expression that is seen following the iPSC differentiation and the resulting effects from high glucose "specific" for endothelia cells? Were parallel studies performed in other cell types derived from iPSC to determine the specificity of the observed findings.

2) Studies done with varying concentrations should include an isoosmolar control with L-glucose being perhaps the best option though mannitol can also be used. If I missed this control, my apologies.

3) I like the approach taken by the authors in regards to the identification of the splicing factor. While this approach could be viewed as too targeted, I think it is reasonable. What I would like to better understand is the magnitude of the genes that are differentially spliced by this factor?

4) I like the studies on the cell-cell interaction protein expression being linked to function though I am less familiar with the system used vs. more conventional studies such as dextran permeability and electrical resistance, A bit more reference situation may be adequate here to limit this concern,

5) While I appreciate the complexity of creating human derived iPSC the authors appear to be using an n=1 for the comparison of the human correlation of diabetes vs. no diabetes.

6) The hind-limb ischemia data while consistent with the hypothesis also may need a bit more attention. More data on the specificity of the lentivirus for hitting endothelial cells vs. other cell types is needed. The general statement is that intramuscular delivery/injection of almost anything hits endothelial cells at a low frequency. This does tie back to one of the prior comments about specificity of the quaking 7 dysregulation in other cell types and the possibiliuty that some of the effects are from cells other than EC.

Reviewer #2:
Remarks to the Author:
This interesting study investigates the role of QKI7 in diabetic vascular disease. In the first part, the authors used iPS-EC (exposed to high glucose) from mice or diabetic patients to evaluate the related signalling and function. They then assessed the potential targets and interactors and finally used in vivo models of ischemia where iPS-ECs expressing the protein were injected or the protein was silencing.
The study provides novel evidence that QKI7 modulates vascular processes. The authors have used novel technologies in their study and the experimental work seems to be well performed and illustrated with details enough for reproduction.

Major comments
Although the cellular model is a sophisticated and certainly useful for screening, it would be certainly important to validate the upregulation of the RBP in endothelial cells and VSMCs from diabetic mice. Even more convincing would be to evaluate the RBP in leftover material from amputated limb of diabetic patients. Contact of potential collaborators providing such a material

can be provided to the authors if they wish to test this in human samples.

The source of human iPS should be clarified as they generically state are from type 1 and 2 diabetic patients without specifying the line where the main results are shown. How do they explain the persistence of the RBP upregulation after iPS generation and endothelial cell differentiation? Did the high glucose increase the expression of the RBP in the human cells? Similarly, do commercial human endothelial cells exposed to high glucose increase the RBP expression?

The in vivo model data are limited to the assessment of blood flow but there is apparently and surprisingly no data about vascular histology, including capillary density, arteriole density, state of myocytes etc so it is not possible to determine what is the structural basis of the perfusion effects and the cellular target of the injected factors.

The conclusion of this being a therapeutic avenue is premature particularly in the light of the lack of experimental evidence in the in vivo studies showing only perfusion.

It is also noted that authors used cells from diabetic donors type 1 and 2 or murine cells exposed to high glucose and then in vivo studies in type 1. High glucose is not the only factor responsible for diabetic complication, further osmotic controls are necessary. Diabetes type 1 and 2 are different pathologies, the authors should focus on one of the pathology and be consistent through the study in the use of a corresponding model.

In conclusion, this is an interesting study but my enthusiasm is reduced by some flaws in the logic connection of the work, the overestimation of glucose induced effect in relation to a diabetic condition, the rationale to use derived cells instead of freshly isolated material and limitations of the in vivo study.

Reviewer #3:
Remarks to the Author:
The topic of the manuscript is clinically and scientifically significant. Microvascular dysfunction is a key process underlying the development of the cardiovascular complications of diabetes. In this manuscript the authors present a detailed study on the role of an isoform of the RNA binding protein Quaking in endothelial function and dysfunction in conditions of hyperglycemia.
The authors report that when using induced pluripotent stem cell derived endothelial cells from mice are exposed to hyperglycemia the QKI-7 isoform is markedly upregulated and that the same holds true for untreated (hyperglycemia) human diabetes patient derived iPS-EC.
Subsequent studies suggest that a shift in the balance of two potential RNA binding slicing factors drives the increase in QKI-7 and that the mechanism involves three genes involved in endothelial integrity, permeability and monocyte adhesion. When QKI-7 is suppressed in iPS-EC and these are infused in a murine ischemic model (and matrigel plugs) the cells promote “angiogenesis”
The final conclusion is that QKI-7 plays a role in diabetic endothelial dysfunction and that the molecule could be a therapeutic tragic to counteract cardiovascular complications of this disease
In most cells QKI-5 goes up first and than, at higher concentrations impacting on the splicing of its own transcript yielding more QKI-6 and 7. The impact of hyperglycemia of the relative regulation of the isoforms is an intriguing observation.

Major comments

While the conclusions drawn seem supported by the directions in the experiments in general the effects are moderate and it is often unclear how many (what is the n of the data?) and whether

there are technical or biological duplicates. Also in several experiments it is unclear in which cells the experiments were performed. E.g. figure 5, 7D how many? Representative pictures?

For instance, the conclusion can be drawn that human iPS-EC from diabetic patients express quite increased levels of QKI-7. Yet, this was shown for two iPS-EC lines from diabetes patients and two control human subjects. A true conclusion on the general applicability of this conclusion might need analysis of multiple cell lines.

Also the source of the diabetes derived iPS-EC is not described other than "patients with type 1 or 2 DM that has the disease over 15 years". However, these diabetes types are quite different and can have an impact on the genetic background of the cells. Could this be more specified.

The impact of hyperglycemia on the mouse cultures iPS-EC expression of QKI-7 is quite strong. It is somewhat unclear how hyperglycemia impacts on the RNA binding proteins CUG-BP and hnRNPM. What is the evidence that these RNA binding proteins physically bind to the QKI precursor RNA. It can not be excluded that overexpressing CUG-BP or hnRNPM affects genes upstream of QKI expression. Mutations for these RBPs in the QKI precursor could substantiate these conclusions.
Ischemic hindlimb models usually mostly assess arteriogenesis much more than angiogenesis. These are different processes and should be taken into account

Minor

QKI-7 is known as a stabilizing factor on genes by binding to the 3' UTR and poly A binding protein. Here QKI-7 is destabilizing the expression of three genes. This could be discussed.

It is surprising that in figure 6C and D overexpression of a single gene that was down regulated by overexpression fully compensates for the loss of function. It can be assumed that multiple factor would be involved. Can this be explained.

Would be informative to note in the introduction that QKI-7 is predominantly cytoplasmic..not directly involved in splicing.

Figure 7A. How many plugs were analysed? Throughout the manuscript the n is problematic..and it is not defined what the n is biological vs technical controls

Targeting QKI-7 in vivo Restores Endothelial Cell Function in Diabetes

3rd December 2019

Response to the Reviewers' comments

We greatly appreciate the reviewers' constructive comments and suggestions. We have appropriately revised our manuscript by including data from new experiments while also addressing all the comments and suggestions.

1. In particular we have provided further experimental data to support the proposed mechanism and clearly shown that the splicing factors CUG-BP and hnRNPM have binding motifs on QKI-7. Interestingly, when human fibroblasts were stimulated with high glucose, neither QKI-7 nor the splicing factors CUG-BP and hnRNPM were induced. These findings highlight the specificity of our proposed mechanisms in ECs in diabetic conditions.

2. Moreover, we have tested the QKI-7 expression 4 additional diabetic iPS-ECs, and we have clearly explained that **ALL** the experiments shown in this study have been validated in 2 independent sets of non-diabetic versus diabetic iPS-ECs (age and sex matched) based on biological replicates with n=3. More details of the donors and the source of iPS cells have been provided.

3. Importantly, based on the reviewers' suggestions we have also validated the QKI-7 high expression in additional human diabetic models; QKI-7 is highly expressed in human coronary arterial ECs isolated from diabetic donors, while immunohistochemical staining on blood vessels obtained from diabetic critical limb ischemia patients undergoing a lower limb amputation, QKI-7 expression was also detected. These findings further underline the central role of QKI-7 in the regulation of EC pathology under diabetic conditions.

4. Additional data based on H&E has been provided, which clearly showing increased angiogenesis / arteriogenesis in diabetic limbs when QKI-7 was knockdown in ECs *in vivo*.

5. The specificity of the lentiviral constructs to target ECs in vivo has also been clearly shown.

6. All the controls have also been fully explained throughout this manuscript.

A detailed response letter to the reviewers' comment is shown below.

Reviewer #1 (Remarks to the Author):

In general terms the paper by Yang et al proposes the finding which is novel that the RNA binding protein Quaking 7 is up-regulated within the diabetic conditions and when up-regulated by a likely splicing factor impairs endothelial cell function in a manner that is consistent with the mechanism of diabetes induced vascular injury. I suggest the authors consider the following suggesting/questions/areas for clarification.

1) Is the increase in Quaking 7 expression that is seen following the iPSC differentiation and the resulting effects from high glucose "specific" for endothelial cells? Were parallel studies performed in other cell types derived from iPSC to determine the specificity of the observed findings.

Response: Thank you for raising this important point. Indeed, we have recently reported that during iPS cells differentiation toward vascular smooth muscle cells only QKI-6 splicing isoform is induced *(Caines, R., et al. The RNA-binding protein QKI controls alternative splicing in vascular cells, producing an effective model for therapy. Journal of cell science **132**(2019),* and neither other QKI isoforms nor QKI-7. In addition, in this manuscript, we have now including additional data showing that when human Fibroblasts were stimulated with high glucose neither QKI-7 nor the splicing factors hnRNPM and CUG-BP were

induced (Supplementary Figure S4 A-B). This data determine the specificity of the observed finding in our manuscript.

2) Studies done with varying concentrations should include an isoosmolar control with L glucose being perhaps the best option though mannitol can also be used. If I missed this control, my apologies.
Response: We apologise for not making this clear in the manuscript. Indeed, L-glucose was used as a control. In the methods and Materials section and in the Figure legends in our revised manuscript this information is clearly now shown.

3) I like the approach taken by the authors in regards to the identification of the splicing factor. While this approach could be viewed as too targeted, I think it is reasonable. What I would like to better understand is the magnitude of the genes that are differentially spliced by this factor?
Response: We have now included additional information in the discussion clearly showing the magnitude of the genes that are differentially spliced by this factor including genes related to epithelial–mesenchymal transition (EMT), and TGFβ signalling. Importantly, hnRNPM competitively binds to G/U rich motifs and regulates genes related to cell adhesion, migration and cytoskeleton organization. This information reflects on the importance of this splicing factor on the regulation of the EC function.

4) I like the studies on the cell-cell interaction protein expression being linked to function though I am less familiar with the system used vs. more conventional studies such as dextran permeability and electrical resistance, A bit more reference situation may be adequate here to limit this concern.
Response: Thank you for this constructive comment. Further information on the Cell barrier examination methods has now been added in the methods and in the results in this manuscript. In particular iPS-ECs were seeded on type IV mouse collagen (5 µg/ml; R&D Systems 3410-010-01)-coated E-Plate VIEW 16 (ACEA Biosciences, Inc. 300600890) in EGM2 medium containing 25ng/ml VEGF at 40,000 cells/well. Continuous monitor was performed overnight using xCELLigence RTCA DP (ACEA Biosciences, Inc.) in a standard CO2 cell culture incubator until the cell index (CI) reached plateau, which represented the impedance of electron flow caused by adherent ECs. At this stage, the culture medium was replaced by OptiMEM to starve the cells for 2 hours. Culture medium was subsequently changed to normal EGM2 supplemented with 50ng/ml VEGF and real-time CI monitoring continued for 24 hours. Cell barrier properties of iPS-ECs were investigated using the Real-Time Cell Analysis (RTCA) with the xCELLigence system. This technology measures cell adhesion using high-density gold electrode arrays in a non-invasive, label-free manner. The iPS-ECs were cultured on the E-Plate with gold electrodes on the bottom of wells which are responsible for the completion of electric circuit of the applied electric potential. The impedance reflected the cell-cell interaction of adherent cells. The RTCA software converted impedance values into the Cell Index (CI), which serves as a readout of cell adhesion.

5) While I appreciate the complexity of creating human derived iPSC the authors appear to be using an n=1 for the comparison of the human correlation of diabetes vs. no diabetes.
Response: Thank you for this comment. In this study, we have repeated ALL the experiments in 4 iPS cells lines from 2 different non-diabetics versus 2 different diabetic donors (sex and age matched) (Supplemental Fig. S6). Furthermore the expression levels of

QKI-7 were also tested in 4 additional diabetic iPS-EC lines verified its high expression in diabetic conditions. Importantly, the QKI-7 high expression was also detected in human coronary ECs isolated from diabetic patients (Supplemental Fig. S7A), while QKI-7 was further increased upon high glucose stimulation (Supplemental Fig. S7B). Remarkably, QKI-7 expression was also detected in the blood vessels of diabetic critical limb ischemia patients (Supplemental Fig. S7C). This information and additional data is now shown in the manuscript, which clearly further underline the central role of QKI-7 in the regulation of critical EC related genes, which impair EC function under diabetic conditions.

6) The hind-limb ischemia data while consistent with the hypothesis also may need a bit more attention. More data on the specificity of the lentivirus for hitting endothelial cells vs. other cell types is needed. The general statement is that intramuscular delivery/injection of almost anything hits endothelial cells at a low frequency. This does tie back to one of the prior comments about specificity of the quaking 7 dysregulation in other cell types and the possibility that some of the effects are from cells other than EC.

Response: This is an important point. In this study, we have generated a shQKI-7 vector under the CD144 Promoter (pLV[miR30-shRNA]-Cd144>EGFP) to target ECs *in vivo*. Since these lentiviral constructs were also tagged with a GFP, the specificity of targeting ECs *in vivo* is shown in Figure 7E, where GFP staining was detected in cells expressing in parallel the EC marker CD144.

Reviewer #2 (Remarks to the Author):

This interesting study investigates the role of QKI7 in diabetic vascular disease. In the first part, the authors used iPS-EC (exposed to high glucose) from mice or diabetic patients to evaluate the related signalling and function. They then assessed the potential targets and interactors and finally used in vivo models of ischemia where iPS-ECs expressing the protein were injected or the protein was silencing. The study provides novel evidence that QKI7 modulates vascular processes. The authors have used novel technologies in their study and the experimental work seems to be well performed and illustrated with details enough for reproduction.

Major comments

1) Although the cellular model is a sophisticated and certainly useful for screening, it would be certainly important to validate the upregulation of the RBP in endothelial cells and VSMCs from diabetic mice. Even more convincing would be to evaluate the RBP in leftover material from amputated limb of diabetic patients. Contact of potential collaborators providing such a material can be provided to the authors if they wish to test this in human samples.

Response: We have followed the important advice of this reviewer and we have performed additional experiments. In particular, the QKI-7 high expression was detected in human coronary arterial ECs isolated from diabetic patients (Supplemental Fig. S7A), while QKI-7 was further increased upon high glucose stimulation in these primary human diabetic cells (Supplemental Fig. S7B). Remarkably, QKI-7 expression was also detected in the blood vessels of diabetic critical limb ischemia patients (Supplemental Fig. S7C). This information and additional data is now shown in the manuscript, which clearly further underline the important role of QKI-7 in the regulation of critical EC related genes, which impair EC function under diabetic conditions.

2) The source of human iPS should be clarified as they generically state are from type 1 and 2 diabetic patients without specifying the line where the main results are shown. How do they

explain the persistence of the RBP upregulation after iPS generation and endothelial cell differentiation? Did the high glucose increase the expression of the RBP in the human cells? Similarly, do commercial human endothelial cells exposed to high glucose increase the RBP expression?

Response: More information on the source of human iPS has been provided in the manuscript.

The point regarding the persistence of the RBP upregulation after iPS generation and endothelial cell differentiation is very interesting. Therefore, the persistence of QKI-7 upregulation after iPS generation and endothelial cell differentiation may be due to **"metabolic memory"** of epigenetic changes under oxidative stress induced by hyperglycaemia (*Togliatto G, Dentelli P, Brizzi MF. Skewed Epigenetics: An Alternative Therapeutic Option for Diabetes Complications. J Diabetes Res. 2015;2015:373708. doi: 10.1155/2015/373708. Epub 2015 Apr 30).* Moreover, the role of epigenetic modifications in diabetes progress continues even after hyperglycemia has been terminated so that intensive glycaemic control cannot effectively protect diabetes patients from vascular complications *(Diabetes Care. 2014 Jan; 37(1): 9–16.).* It has also been shown that the long term condition of hyperglycaemia has significant effects on the epigenome leading to aberrant chromatin modification and gene expression associated with diabetic vascular endothelial dysfunctions *(Jin J, Wang X, Zhi X, Meng D. Epigenetic regulation in diabetic vascular complications. J Mol Endocrinol. 2019 Oct 1.).* There is ample evidence that reprogrammed iPSCs maintain epigenetic signatures inherited from initial somatic cells *(Vaskova EA, Stekleneva AE, Medvedev SP, Zakian SM. "Epigenetic memory" phenomenon in induced pluripotent stem cells. Acta Naturae. 2013 Oct;5(4):15-21).* **In this way, pathogenic epigenetic modification in patient donor cells may be passed on through iPSCs, resulting in consistent dysregulation of genes associated with diabetic endothelial dysfunction including QKI-7.**

To completely satisfy this reviewer's comment, we have conducted further experiments based on the 2 suggested approaches that the reviewer proposed: 1. *Did the high glucose increase the expression of the RBP in the human cells? 3. Similarly, do commercial human endothelial cells exposed to high glucose increase the RBP expression?*
As it has been shown above, human coronary arterial ECs isolated from diabetic patients were used and compared with arterial ECs isolated from non-diabetic donors, or human ECs were further exposed to glucose stimulation. In all these cases, QKI-7 expression was induced and this data is now shown in the supplementary Figures S7A and B.

3) The in vivo model data are limited to the assessment of blood flow but there is apparently and surprisingly no data about vascular histology, including capillary density, arteriole density, state of myocytes etc so it is not possible to determine what is the structural basis of the perfusion effects and the cellular target of the injected factors. The conclusion of this being a therapeutic avenue is premature particularly in the light of the lack of experimental evidence in the in vivo studies showing only perfusion.

Response: Thank you for this constructive comment. These experiments have now been performed and the data is shown in the Figure 7D.

4) It is also noted that authors used cells from diabetic donors type 1 and 2 or murine cells exposed to high glucose and then in vivo studies in type 1. High glucose is not the only factor responsible for diabetic complication, further osmotic controls are necessary. Diabetes type 1 and 2 are different pathologies, the authors should focus on one of the pathology and be consistent through the study in the use of a corresponding model.

Response: We totally agree that high glucose is not the only factor responsible for diabetic complications. We have now made it clear that long term diabetic patients have been recruited in this study, as we have generated iPS cells from diabetes patients who have been diagnosed with the disease for more than 15 years. The key characteristic of these patient specific iPS-ECs was EC dysfunction in terms of angiogenesis, neovasculisation and permeability. We have validated that QKI-7 is also expressed in primary cardiac arterial ECs from long term diabetic patients and in blood vessels from diabetic critical limb ischemia patients. Regarding the comment with the osmotic controls, L-glucose control has been used for all the glucose experiments. This information, it is clearly shown in the manuscript.

5) In conclusion, this is an interesting study but my enthusiasm is reduced by some flaws in the logic connection of the work, the overestimation of glucose induced effect in relation to a diabetic condition, the rationale to use derived cells instead of freshly isolated material and limitations of the in vivo study.

Response: We trust that the revised manuscript, the new data and our responses have addressed all your comments. Indeed, we have validated the findings of our study **in freshly isolated materials** and samples from critical limb ischemia patients undergoing a lower limb amputation. It is also important to highlight that iPS cells provide the tools to generate unlimited numbers of diabetic ECs that capture the phenotype of the disease in a petri dish. This approach allows the investigation of the underlying mechanisms such as QKI-7 to be performed using an attractive human diabetic model. Regarding the *in vivo* model, we have combined a mouse diabetic model with the limb hind ischemia model, which is highly correlated with diabetes. We have clearly shown that targeting QKI-7 *in vivo* restores EC function in diabetic mice by improving blood flow in the ischemic diabetic limbs.

Reviewer #3 (Remarks to the Author):

The topic of the manuscript is clinically and scientifically significant. Microvascular dysfunction is a key process underlying the development of the cardiovascular complications of diabetes. In this manuscript the authors present a detailed study on the role of an isoform of the RNA binding protein Quaking in endothelial function and dysfunction in conditions of hyperglycemia. The authors report that when using induced pluripotent stem cell derived endothelial cells from mice are exposed to hyperglycemia the QKI-7 isoform is markedly upregulated and that the same holds true for untreated (hyperglycemia) human diabetes patient derived iPS-EC. Subsequent studies suggest that a shift in the balance of two potential RNA binding slicing factors drives the increase in QKI-7 and that the mechanism involves three genes involved in endothelial integrity, permeability and monocyte adhesion. When QKI-7 is suppressed in iPS-EC and these are infused in a murine ischemic model (and matrigel plugs) the cells promote “angiogenesis” The final conclusion is that QKI-7 plays a role in diabetic endothelial dysfunction and that the molecule could be a therapeutic tragic to counteract cardiovascular complications of this disease. In most cells QKI-5 goes up first and than, at higher concentrations impacting on the splicing of its own transcript yielding more QKI-6 and 7. The impact of hyperglycemia of the relative regulation of the isoforms is an intriguing observation.

Major comments

1) While the conclusions drawn seem supported by the directions in the experiments in general the effects are moderate and it is often unclear how many (what is the n of the data?) and whether there are technical or biological duplicates. Also in several experiments it is

unclear in which cells the experiments were performed. E.g. figure 5, 7D how many? Representative pictures?
Response: All the information regarding the representative images and n of the data are now clearly described in the revised manuscript. In this study, for all the experiments biological replicates were performed with n=3.

2) For instance, the conclusion can be drawn that human iPS-EC from diabetic patients express quite increased levels of QKI-7. Yet, this was shown for two iPS-EC lines from diabetes patients and two control human subjects. A true conclusion on the general applicability of this conclusion might need analysis of multiple cell lines.
Response: Thank you for this comment, as we have responded above; In this study, we have repeated ALL the experiments in 4 iPS cells lines from 2 different non-diabetics versus 2 different diabetic donors (Supplemental Fig. S6). Furthermore the expression levels of QKI-7 were also tested in 4 additional diabetic iPS-EC lines verified its high expression in diabetic conditions. Importantly, the QKI-7 high expression was detected in human coronary ECs isolated from diabetic patients (Supplemental Fig. S7A), while QKI-7 was further increased upon high glucose stimulation (Supplemental Fig. S7B). Remarkably, QKI-7 expression was also detected in the blood vessels of diabetic critical limb ischemia patients (Supplemental Fig. S7C). This information and additional data is now shown in the manuscript, which clearly further underline the important role of QKI-7 in the regulation of critical EC related genes, which impair EC function under diabetic conditions.

3) Also the source of the diabetes derived iPS-EC is not described other than "patients with type 1 or 2 DM that has the disease over 15 years". However, these diabetes types are quite different and can have an impact on the genetic background of the cells. Could this be more specified.
Response: More information on the source of the diabetes derived iPS-EC is now provided in the revised manuscript.

4) The impact of hyperglycemia on the mouse cultures iPS-EC expression of QKI-7 is quite strong. It is somewhat unclear how hyperglycemia impacts on the RNA binding proteins CUG-BP and hnRNPM. What is the evidence that these RNA binding proteins physically bind to the QKI precursor RNA. It can not be excluded that overexpressing CUG-BP or hnRNPM affects genes upstream of QKI expression. Mutations for these RBPs in the QKI precursor could substantiate these conclusions.
Response: In the Supplemental Figure S3 we provide evidence that there are predicted RNA binding sites for CUG-BP and hnRNPM within QKI-7 sequence, in the Supplemental Figure S3A it is shown predicted RNA binding motifs of CUG-BP located at the intron 6-exon 7 interface district of QKI-7 gene sequence, while in the Supplemental Figure S3B, the predicted RNA binding motifs of hnRNPM located within the intron 6 district of QKI-7 gene sequence are shown. Interestingly, we have now included additional data in this manuscript showing that when human Fibroblasts were stimulated with high glucose neither QKI-7 nor the splicing factors hnRNPM and CUG-BP were induced (Supplementary Figure S4A and B). This data determine the specificity of the observed findings in our manuscript.

5) Ischemic hindlimb models usually mostly assess arteriogenesis much more than angiogenesis. These are different processes and should be taken into account.

Response: Thank you for this comment. We agree with the reviewer that arteriogenesis is also assessed in the Ischemic hindlimb models. This discussion has now been added in the revised manuscript.

Minor

6) QKI-7 is known as a stabilizing factor on genes by binding to the 3' UTR and poly A binding protein. Here QKI-7 is destabilizing the expression of three genes. This could be discussed.

Response: This has now been discussed in the introduction and discussion of the revised manuscript.

7) It is surprising that in figure 6C and D overexpression of a single gene that was down regulated by overexpression fully compensates for the loss of function. It can be assumed that multiple factor would be involved. Can this be explained. Would be informative to note in the introduction that QKI-7 is predominantly cytoplasmic..not directly involved in splicing.
Figure 7A. How many plugs were analysed? Throughout the manuscript the n is problematic..and it is not defined what the n is biological vs technical controls.

Response: All these minor points have now been discussed and addressed in the revised manuscript.

Thank you once again for your constructive comments, critical approach and helpful suggestions, which have substantially improved our manuscript. We are confident that we have addressed all the comments and this revised manuscript clearly represents the central role of QKI-7 in the pathology of ECs in diabetes.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
I have no additional comments/questions.

Reviewer #2:
Remarks to the Author:
The manuscript is substantially improved, but the study of angiogenesis is still not complete and questionable. In Figure 7D the authors used HE to count capillaries. They report values of capillaries that are very low compared with the expected cap in the murine muscle (5 to 7 hundreds caps/mm2). Is the number reported 5 to 10 cap/mm2 this would be low even for arterioles.

Moreover this figure refers only to the analysis of the exp with silencing but not to the one with iPS. Both need to be assessed. In doing so, they should use a specific marker for endothelial cells and SMCs such as CD31/lectin and aSMA respectively. Then use morphometry.

Moreover, the authors did not performed the suggestion to study proliferation and apoptosis, therefore it is not clear from where the change in vascularity derives.

Reviewer #3:
Remarks to the Author:

I asked for evidence of physical binding of the RBP to the QKI-7 transcriptt. Other than identfication of potential binding sites this was not provided. Ergo this remains speculative

minor:

In bottom abstract grammer make e.g. "QKI-7 was knocked down...

Also the rebuttal says experiments are now performed with 4 different ips-EC but fig S6 and 7 only refer to HD22 and PO23 did I look at the revised figures?

Targeting QKI-7 in vivo Restores Endothelial Cell Function in Diabetes

20th March 2020

Response to the Reviewers' comments

We greatly appreciate the reviewers' constructive comments and suggestions. We appropriately revised our manuscript by including data from new experiments.

Reviewer #1 (Remarks to the Author): I have no additional comments/questions.

Response: Many thanks for your constructive comments and your time reviewing our manuscript.

Reviewer #2 (Remarks to the Author): The manuscript is substantially improved, but the study of angiogenesis is still not complete and questionable. In Figure 7D the authors used HE to count capillaries. They report values of capillaries that are very low compared with the expected cap in the murine muscle (5 to 7 hundreds caps/mm2). Is the number reported 5 to 10 cap/mm2 this would be low even for arterioles. Moreover this figure refers only to the analysis of the exp with silencing but not to the one with iPS. Both need to be assessed. In doing so, they should use a specific marker for endothelial cells and SMCs such as CD31/lectin and aSMA respectively. Then use morphometry. Moreover, the authors did not performed the suggestion to study proliferation and apoptosis, therefore it is not clear from where the change in vascularity derives.

Response: Thank you for your constructive comments and for your reviewing our manuscript. To address these comments, we performed additional experiments and further analysis. Specifically, 1. We revised the Figure 7D and we now include data showing not only the capillary density (Figure 7D) but also the arterioles and venule density (Supplemental Figure S9). 2. Hematoxylin and Eosin staining of iPS-ECs overexpressing QKI-7 is also shown confirming a significant suppression in *in vivo* angiogenic capacity in comparison to iPS-ECs overexpressing a vehicle control (Supplemental Figure S8). 3. HRP-DAB immunohistochemical staining with CD31 on the sections injected with lentiviral shQKI-7 (KD) showing increased arterioles and venule density, and HRP-DAB immunohistochemical staining for the smooth muscle marker SMA are also included in the revised manuscript (Supplemental Figure S9). In addition, we had performed migration and proliferation assays and QKI-7 does not have an effect, however the precise role and mechanism of the QKI-7 in diabetic vasculopathy is summarized in Supplemental Figure S11.

Reviewer #3 (Remarks to the Author):
I asked for evidence of physical binding of the RBP to the QKI-7 transcript. Other than identification of potential binding sites this was not provided. Ergo this remains speculative.

Response: Thank you for your constructive comments and for your reviewing our manuscript. To fully address this comment, we have now performed RNA immunoprecipitation assays, which clearly prove the physical binding of the RBP proteins (CUG-BP and hnRNPM) to the QKI-7 transcript. (The data is shown in the supplemental Figure S3).

Thank you once again for your constructive comments, critical approach and helpful suggestions, which have substantially improved our manuscript. We are confident that we have addressed all the comments and this revised manuscript clearly represents the central role of QKI-7 in the pathology of ECs in diabetes.

Thank you for your consideration.

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
I do not have any additional question

Reviewer #3:
Remarks to the Author:
The RIPs look good and now provide direct evidence for binding to the transcript

Targeting QKI-7 in vivo Restores Endothelial Cell Function in Diabetes

25th May 2020

Response to the Reviewers' comments

We greatly appreciate the reviewers' positive responses.

REVIEWERS' COMMENTS:

Reviewer #2 (Remarks to the Author):

I do not have any additional question

Response: We are grateful that the reviewer is fully satisfied of our study.

Reviewer #3 (Remarks to the Author):

The RIPs look good and now provide direct evidence for binding to the transcript

Response: We are very pleased that the reviewer is fully satisfied of the RIPs experiments.

Thank you once again for your constructive comments, critical approach and helpful suggestions, which have substantially improved our manuscript.