EMBO reports

Smad4 promotes diabetic nephropathy by modulating glycolysis and OXPHOS

Jinhua Li, Yu Bo Yang Sun, Weiyi Chen, Jinjin Fan, Songhui Li,Xinli Qu, Qikang Chen, Riling Chen, Dajian Zhu, Jinfeng Zhang, Zhuguo Wu , Honggang Chi, Simon Crawford, Viola Oorschot, Victor G. Puelles, Peter G Kerr, Yi Ren, Susan K. Nilsson, Mark Christian, Huanwen Tang, Wei Chen, John F. Bertram, David J. Nikolic-Paterson, Xueqing Yu

Review timeline:

Submission date:	2 July 2019
Editorial Decision:	30 July 2019
Additional Correspondence:	28 August 2019
Editorial Decision:	20 September 2019
Additional Correspondence:	24 September 2019
Editorial Decision:	16 October 2019
Revision received:	31 October 2019
Accepted:	18 November 2019

Editor: Deniz Senyilmaz-Tiebe

Transaction Report:

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

1st Editorial Decision 30 July 2019

Thank you for submitting your manuscript entitled 'Smad4 modulates glycolysis and OXPHOS via interactions with PKM2 and ATPIF1 in diabetic nephropathy' to EMBO Reports. We have now received two referee reports, which are included below. I have also triggered a referee cross-commenting session, where referees comment on each other's reports. In light of these comments and discussions, we unfortunately had to conclude that the study is not a strong candidate for publication in EMBO Reports.

While the referees find the proposed role of Smad4 in modulation of diabetic nephropathy, they also raise concerns that preclude publication of the manuscript in this journal. Referees point out a number of concerns regarding the conclusiveness of the dataset and the methodologies used; therefore do not recommend publication here. Given these comments from recognized experts in the field who are also experienced reviewers, we cannot offer to publish your manuscript. However, in case you feel that you can address the referee concerns in a timely and thorough manner, and can obtain data that would considerably strengthen the study as in the referee reports, we would have no objection to consider a new manuscript (along with a point-by-point response to the referee concerns) on the same topic in the future. Please note that if you were to send a new manuscript this would be assessed again with respect to the literature and the novelty of your findings at the time of resubmission and in case of a positive editorial evaluation, the manuscript would be sent back to the original referees. I would like to emphasize that we need strong support from the referees to consider publication here.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that I cannot communicate more positive news, but nevertheless hope that you will find our referees' comments helpful.

REFEREE REPORTS

Referee #1:

Comments to authors and report: This is a report can be transferred to authors.
The manuscript by Jinhua Li, et al, titled 'Smad4 modulates glycolysis and OXPHOS via interactions with PKM2 and ATPIF1 in diabetic nephropathy' reported that diabetes induced elevation of Smad4 as downstream target of TGF-β1. The new findings are the deletion Smad4 will increase glycolysis and oxidative phosphorylation which reduces reactive oxygen species and prevent high glucose induced glomerular cell damage in vitro and vivo. They also provided new findings of Smad4 bound to PKM2 and decrease the tetrameric forms of PKM2. In addition, they reported that Smad4 also interacted with ATPIF1 which decreased ATP formation and correlated to podocytes injury.
This study provided a large amount of interesting data and could potentially improve the understanding of diabetes induced injury in podocytes and contribute to nephropathy in diabetes.
There are several major concerns:
1. The mice models used are not clear as to whether they are type 1 or type 2 diabetes models since the study uses high fat diet mice which were given STZ to induce insulin deficiency. However these mice in this study still have elevated insulin levels which suggested that the effect of STZ was not significant. It is difficult to understand the cause of diabetes in these mice since high fat diet alone only causes very minor diabetes. Clear idea of diabetic models in animal study is important since the use of wrong mice models will be important to assess the applicability to human diseases. The authors should compare the insulin responses in their model of high fat diet with and without diabetes to determine whether there are any differences in insulin levels and to clarify the pathophysiology of diabetes in these models.
2. Results in Figure 1 are all immunohistology which makes quantitation difficult. It will be very important to evaluate the findings in Figure 1 either at mRNA or protein levels using RT-PCR or Western blotting studies.
3. The study using eNOS-/- Smad4-/- mice on high fat diet mice is a good idea. However the induction of diabetes should have been done in intervention rather than prevention studies. Intervention studies are more reproducible to clinical studies than prevention studies. Many agents have shown efficiency for treatment of diabetes nephropathy or retinopathy but their data has not translated into similar findings in diabetic patients. Whereas intervention studies have had better success. Intervention studies are defined by having the mice with diabetes after at least 2 months before intervention with an agent or a device. Pathology in kidney can be stopped or reversed by experimental interventions.
4. The data in Figure 3 showed that high fat diet plus STZ cause enlarge glomeruli with some improvement in Smad4 minus mice. However the picture showed glomerular size is still much bigger in Smad4-/- mice than the non-diabetic control. The collagen staining in Figure 3G showed a complete reversal. This is clearly unusual since a large glomeruli will have more collagen. This paradox needs to be explained.
5. The Figure 4 was performed only in podocytes Smad4-/- mice and control mice. These studies clearly need to be done with the 4 groups of mice which have been studied in Figure 3. Otherwise it is difficult to understand the rationale and the findings of the studies.
6. A variety of studies were performed with low and high glucose. There is no explanation of the concentration of glucose in low and high glucose levels which needs to be defined.

Confidential comments to editors: This manuscript provided interesting findings and many types of experiment. However the types of studies were not defined in details which I made many recommendations. This manuscript clearly needs to have major revisions before it can be accepted.

Referee #2:

Li et al. investigated the role of Smad4 in diabetes-associated podocyte injury. They discovered that in an experimental model of DN induced by HD+STZ, Smad4 is upregulated and it can modulate glycolysis and oxidative phosphorylation by inhibiting PKM2 activity and stabilizing ATPIF1 through potential protein-protein interaction independent its nuclear localization.

Major concerns:
1) The Results section of this paper needs to be rewritten. It did not do a good job to walk the readers through the data. Did not state the hypothesis for each set of experimental data, did not describe how each experiment was designed and what the results are. The figures and the figure legends don't have sufficient information for me to understand how these experiments were designed and carried out. They merely state the conclusion for each experiment. This is very poorly written!
2) Figure 1: WT1 staining is of low quality, it did not stain the nucleus clearly so how can the authors be using this as a podocyte marker? The mitochondrial staining of Smad4 is not convincing, given that Smad4 appears to have a lot of cytoplasmic staining. The staining of Smad4 is more intense in cells out of glomeruli too, is this true?
3) STZ is toxic to pancreatic beta cells and it is used to induce type 1 diabetes in animal models. Why the experimental model described in the manuscript has hyperinsulinemia? What is the purpose using STZ?
4) Figure 3: PAS staining is used to detect glomerulosclerosis not for detection of mesangial expansion.
5) When Seahorse assays was carried out with isolated mouse podocytes, was normal level of glucose and insulin used in media. Should high glucose and insulin be used to mimic in vivo condition, given that the mice with HD+STZ had hyperglycemia and hyperinsulinemia?
6) Figure 6& 7: PKM2 protein levels are elevated in the DN model, but on Figure 7c, I can't appreciate that total amount of PKM2 is higher in HG than NG. please explain this discrepancy.
7) Figure 9H: I have no clue how this set of experiment is designed and how the data is interpreted.
8) Figure 10: how this experiment was designed and carried out? was HG or NG condition used for this set of experiment?

Minor concerns:

1) Many grammatical errors and typos need to be fixed.
2) The manuscript is poorly organized. e.g. the abstract was poorly written, as it is hard to understand why smad4 was studied by reading the abstract. Another example is that at the beginning of third section in Resutls, two sentences were used to introduce synaptopodin, but I don't understand the purpose of doing this.
3) The results section should be expanded, but many figures can be combined to preserve a suitable length of the manuscript.

Additional Correspondence 28 August 2019

We recently received your decision on our submitted manuscript (EMBOR-2019-48781-T). Based on your decision, we have added an intervention study demonstrating a protective effect of anti-Smad4 locked nucleic acid treatment in established disease which addresses the main point raised by the referees. We have made point-by-point responses to the issues raised by the referees, including a substantial re-write of the manuscript. We consider that this manuscript represents a significant advance in the field of metabolic diseases which will be of general interest to the readers of EMBO Reports. We hope that our manuscript is now acceptable for publication.

Referee #1:

Comments to authors and report: This is a report can be transferred to authors. The manuscript by Jinhua Li, et al, titled 'Smad4 modulates glycolysis and OXPHOS via interactions with PKM2 and ATPIF1 in diabetic nephropathy' reported that diabetes induced elevation of Smad4 as downstream target of TGF-β1. The new findings are the deletion Smad4 will increase glycolysis and oxidative phosphorylation which reduces reactive oxygen species and prevent high glucose induced glomerular
cell damage in vitro and vivo. They also provided new findings of Smad4 bound to PKM2 and decrease the tetrameric forms of PKM2. In addition, they reported that Smad4 also interacted with ATPIF1 which decreased ATP formation and correlated to podocytes injury. This study provided a

large amount of interesting data and could potentially improve the understanding of diabetes induced injury in podocytes and contribute to nephropathy in diabetes. There are several major concerns:

1. The mice models used are not clear as to whether they are type 1 or type 2 diabetes models since the study uses high fat diet mice which were given STZ to induce insulin deficiency. However these mice in this study still have elevated insulin levels which suggested that the effect of STZ was not significant. It is difficult to understand the cause of diabetes in these mice since high fat diet alone only causes very minor diabetes. Clear idea of diabetic models in animal study is important since the use of wrong mice models will be important to assess the applicability to human diseases. The authors should compare the insulin responses in their model of high fat diet with and without diabetes to determine whether there are any differences in insulin levels and to clarify the pathophysiology of diabetes in these models.

Reply: The HFD/STZ protocol is widely used in studies of mice and rats as an accelerated model of type 2 diabetes (see a review by S Skovso. J Diabetes Invest 2014; 5: 349–358 and refs 22-31). This model uses a high fat diet to induce obesity, hyperinsulinemia and glucose intolerance, and then employs a low dose of STZ to induce a substantial (but not total) reduction in functional beta cell mass. Together, these two stressors are designed to mimic the pathology of type 2 diabetes – with many similarities evident between this rodent model and the human condition (S Skovso. J Diabetes Invest 2014; 5: 349–358). Our HFD/STZ protocol in eNOS-/- mice resulted in increased body weight, increased plasma insulin levels, glucose intolerance and elevated HbA1c – all features of type 2 diabetes.

2. Results in Figure 1 are all immunohistology which makes quantitation difficult. It will be very important to evaluate the findings in Figure 1 either at mRNA or protein levels using RT-PCR or Western blotting studies.

Reply: Immunostaining in Figure 1 was performed on 4um sections of formalin-fixed tissue from needle biopsy specimens of human kidney. This approach was chosen to localise protein expression to individual cell types. However, 4um sections of biopsy specimens are not sufficient, or suitable, for Western blotting. While RT-PCR analysis can be performed on biopsy sections, this would only provide an average Smad4 mRNA level across all of the different cell types in the section.

3. The study using eNOS-/- Smad4-/- mice on high fat diet mice is a good idea. However the induction of diabetes should have been done in intervention rather than prevention studies. Intervention studies are more reproducible to clinical studies than prevention studies. Many agents have shown efficiency for treatment of diabetes nephropathy or retinopathy but their data has not translated into similar findings in diabetic patients. Whereas intervention studies have had better success. Intervention studies are defined by having the mice with diabetes after at least 2 months before intervention with an agent or a device. Pathology in kidney can be stopped or reversed by experimental interventions.

Reply: We agree with this point. Since it is not possible to perform an intervention study in mice with permanent Smad4 gene deletion in podocytes, we have included an intervention study using a different strategy. This study aimed to down-regulate Smad4 expression during the last 6 weeks of the 30-week HFD/STZ model. This was achieved by systemic administration of an anti-Smad4 locked nucleic acid (LNA-Smad4) which is preferentially taken up by the kidney. As shown in the new Fig 1, administration of LNA-Smad4 significantly decreased Smad4 protein levels in the kidney (but not in spleen, liver or lung) and halted progression of DN. One limitation of this study is that we cannot solely attribute the protective effect of LNA-Smad4 treatment to blocking Smad4 actions in podocytes since Smad4 expression was reduced in the kidney as a whole. However, this LNA-Smad4 study establishes the therapeutic potential of targeting Smad4 in established DN.

4. The data in Figure 3 showed that high fat diet plus STZ cause enlarge glomeruli with some improvement in Smad4 minus mice. However the picture showed glomerular size is still much bigger in Smad4-/- mice than the non-diabetic control. The collagen staining in Figure 3G showed a complete reversal. This is clearly unusual since a large glomeruli will have more collagen. This paradox needs to be explained.

Reply: The induction of hyperglycaemia causes glomerular hypertrophy as pointed out by the reviewer. We measured glomerular collagen IV staining as a percentage of the total glomerular tuft area – making this measurement independent of the glomerular volume. Thus, while a large glomerulus will have more collagen than a small glomerulus, the percentage area of collagen

staining in the tuft need not be different. The data in Fig 3G shows that Smad4 deletion in podocytes prevents (not a reversal) an increase in the percentage area of the glomerular tuft with collagen IV staining in the DN model.

5. The Figure 4 was performed only in podocytes Smad4-/- mice and control mice. These studies clearly need to be done with the 4 groups of mice which have been studied in Figure 3. Otherwise it is difficult to understand the rationale and the findings of the studies.
Reply: As requested, we have added data for Smad4-/- and WT podocytes cultured under normal and high glucose conditions in testing the effect of Smad4 on mitochondrial function (new Fig 4A-D). This represents the groups of diabetic and non-diabetic mice, with and without podocyte Smad4 deletion, examined in the DN model. In addition, we have combined data from Figs 3 and 4 into Fig 4 in the revised manuscript to make the study easier to follow.

6. A variety of studies were performed with low and high glucose. There is no explanation of the concentration of glucose in low and high glucose levels which needs to be defined.

Reply: We used standard conditions for high glucose (4.5g/L D-glucose) and normal glucose (1g/L D-glucose) in the cell culture studies. This has been specified in both the text and in the methods section.

Confidential comments to editors: This manuscript provided interesting findings and many types of experiment. However the types of studies were not defined in details which I made many recommendations. This manuscript clearly needs to have major revisions before it can be accepted.

Reply: We have added the LNA-Smad4 intervention study to the manuscript. The manuscript has also been re-structured and re-written to improve clarity.

Referee #2:

Li et al. investigated the role of Smad4 in diabetes-associated podocyte injury. They discovered that in an experimental model of DN induced by HD+STZ, Smad4 is upregulated and it can modulate glycolysis and oxidative phosphorylation by inhibiting PKM2 activity and stabilizing ATPIF1 through potential protein-protein interaction independent its nuclear localization.

Major concerns:
1) The Results section of this paper needs to be rewritten. It did not do a good job to walk the readers through the data. Did not state the hypothesis for each set of experimental data, did not describe how each experiment was designed and what the results are. The figures and the figure legends don't have sufficient information for me to understand how these experiments were designed and carried out. They merely state the conclusion for each experiment. This is very poorly written!
Reply: We were very conscious of length of the manuscript. However, we have re-written the manuscript in a more expansive fashion to provide greater clarity.

2) Figure 1: WT1 staining is of low quality, it did not stain the nucleus clearly so how can the authors be using this as a podocyte marker? The mitochondrial staining of Smad4 is not convincing, given that Smad4 appears to have a lot of cytoplasmic staining. The staining of Smad4 is more intense in cells out of glomeruli too, is this true?
Reply: The WT1 antibody used recognizes an isoform of WT1 present in the cytoplasm, not in the nucleus, of podocytes (39, 40). This point has been added to the revised text.
The reviewer is correct that Smad4 is present in both the cytoplasm and mitochondria in podocytes. Figure 1 clearly shows a marked increase in Smad4 protein levels in both the cytoplasm (co-localised with WT-1) and in mitochondria (co-localised with Tom20) in kidneys from patients with DN compared to control normal human kidney.
Smad4 is expressed by most cells in the kidney. Tubular epithelial cells express high levels of Smad4.

3) STZ is toxic to pancreatic beta cells and it is used to induce type 1 diabetes in animal models. Why the experimental model described in the manuscript has hyperinsulinemia? What is the purpose using STZ?
Reply: Please see the reply to Referee #1. In addition, we wish to emphasize that the model uses a single low dose of STZ which does not kill all insulin producing beta cells, thereby enabling the state of hyperinsulinemia to be maintained.

4) Figure 3: PAS staining is used to detect glomerulosclerosis not for detection of mesangial expansion.
Reply: The reviewer is correct; the text was in error. It should have stated, "… including mesangial matrix expansion (PAS staining) …". This part of the text has been revised.

5) When Seahorse assays was carried out with isolated mouse podocytes, was normal level of glucose and insulin used in media. Should high glucose and insulin be used to mimic in vivo condition, given that the mice with HD+STZ had hyperglycemia and hyperinsulinemia?
Reply: Many factors, such as hyperglycaemia, hyperinsulinemia, hyperlipidaemia, hypertension and oxidative stress may contribute to the pathogenesis of DN. The Seahorse assays focused on the effect of hyperglycaemia and were performed under conditions of normal and high glucose. All cultures used normal insulin levels.

6) Figure 6& 7: PKM2 protein levels are elevated in the DN model, but on Figure 7c, I can't appreciate that total amount of PKM2 is higher in HG than NG. please explain this discrepancy.
Reply: These are different experiments. Fig 6A and B (now Fig 5A and B) shows an increase in PKM2 levels in the whole diabetic kidney. The study in Fig 7C (now Fig 5L) examines how a period of 24hr of high glucose conditions affects the monomer/dimer/tetramer forms of PKM2 in cultured podocytes. The total PKM2 protein level did not change in this short-term culture study, rather there was a change in PKM conformation.

7) Figure 9H: I have no clue how this set of experiment is designed and how the data is interpreted.
Reply: We have removed this experiment.

8) Figure 10: how this experiment was designed and carried out? was HG or NG condition used for this set of experiment?

Reply: We have provided an expanded explanation for this methodology and conclusions drawn from this experiment.

Minor concerns:

1) Many grammatical errors and typos need to be fixed.
Reply: The grammatical and typographical errors have been corrected.

2) The manuscript is poorly organized. e.g. the abstract was poorly written, as it is hard to understand why smad4 was studied by reading the abstract. Another example is that at the beginning of third section in Results, two sentences were used to introduce synaptopodin, but I don't understand the purpose of doing this.
Reply: We have revised the abstract and substantial portions of the text to make the manuscript easier to follow.

3) The results section should be expanded, but many figures can be combined to preserve a suitable length of the manuscript.
Reply: We have expanded the results text considerably to improve the flow of the manuscript. We have combined some of the Figures as suggested.

2nd Editorial Decision 20 September 2019

Thank you for the submission of your revised manuscript to EMBO reports. It has been sent back to original referees and they have now returned their reports.

As you will see, referee 2 supports publication of the manuscript with minor changes. Referee 1 however remains unconvinced of the animal models and some of the methodologies used.

Given the substantial concerns raised by this referee, the fact that you already had a chance to significantly revise the study and that EMBO Reports allows a single round of revision only, I am afraid that we cannot offer to publish the manuscript at this point.

I am sorry that I could not bring better news this time and hope that the referee comments will be helpful in your continued work in this area.

REFEREE REPORTS

Referee #1:

Comments to the authors
The authors have made some changes. However, they did not answer some simple requests.
1. In regards to the models the authors should at least test whether the animals that have been used with IPGTT or IVITT to measure glucose and insulin levels. Both of these physiological studies are commonly performed in animal study to verify the sensitivity to insulin and the severity of diabetes.
2. This request was made to confirm the data presented in Figure 1 using immunohistological study by quantitative mRNA or protein levels which are the usual methods to confirm immunohistological study. This is done by almost every study in diabetic nephropathy in animal and clinical studies. In addition, the authors should isolate glomeruli to ascertain the changes since the goal is to study diabetes nephropathy. Using urine and whole kidney are clearly not the best way to identify changes in glomeruli and podocytes. Published studies in diabetic nephropathy usually use isolated glomeruli to their studies, especially in rodent studies.

Referee #2:

Li et al. presented the functional study of Smad4 in a murine model of accelerated DN. They discovered that elevated Smad4 expression in the podocytes of the DN animal reduced the tetramer formation of PKM2 and the protein expression of ATPIF1, which subsequently led to the altered energy metabolism. The findings are novel and informative to the field of DN research.
The revised manuscript has a significant improvement of readability.

Minor concerns

1) Please specify what "DN" and "DM" represents. The authors used both abbreviations in the figures, but they were never defined in the legends or in the text.
2) Figure 2D-F are of low quality and pixelated.
3) Please state clearly the sample size when present a statistical analysis.
4) The LNA knockdown study in figure 1 to a large degree is to confirm the conditional KO of Smad4. I don't think this is of critical importance. It may be placed in the supplement to the paper. It feels unnatural to start the paper with this set of data.

Additional Correspondence 23 September 2019

We were surprised by the rejection of our revised manuscript based on the comments of referee 1. We are concerned that we did not receive a fair review. We provide a brief point-by-point reply to referee 1 below and request that our revised manuscript be sent to a third referee.

Referee #1:

The authors have made some changes. However, they did not answer some simple requests.

1. The authors should at least test whether the animals that have been used with IPGTT or IVITT to measure glucose and insulin levels. Both of these physiological studies are commonly performed in animal study to verify the sensitivity to insulin and the severity of diabetes.

Reply: We are puzzled by this comment since this data is already provided in the manuscript. The severity of diabetes is shown by the marked increase in the level of glycated haemoglobin (HbA1c) in the model as shown in Fig 1I, Suppl Fig 1C and Supp Fig 4C (numbering from the revised version); HbA1c is the gold standard method for assessing long term blood glucose control (fasting blood glucose levels are also shown in Supp Fig 4B). In addition, the impairment of the intraperitoneal glucose tolerance test (IPGTT, Suppl Fig 2D-E and Suppl Fig 4E-F) and the substantially elevated plasma insulin levels (Suppl Fig 1D, Suppl Fig 2C & Suppl Fig 4D) in this model are provided.

2. This request was made to confirm the data presented in Figure 1 using immunohistological study by quantitative mRNA or protein levels which are the usual methods to confirm immunohistological study. This is done by almost every study in diabetic nephropathy in animal and clinical studies. In addition, the authors should isolate glomeruli to ascertain the changes since the goal is to study diabetes nephropathy. Using urine and whole kidney are clearly not the best way to identify changes in glomeruli and podocytes. Published studies in diabetic nephropathy usually use isolated glomeruli to their studies, especially in rodent studies.

Reply: We are puzzled by these comments. First, we have yet to see a published paper which has used standard Western blot analysis of human kidney biopsies (as opposed to nephrectomy samples). Second, we agree that it is the analysis of glomerular changes which is critical in studies of diabetic kidney disease. Furthermore, it is damage to the podocyte within the glomerulus which is the critical event in this disease - and the focus of this study. This is why we directly isolated podocytes from the diabetic kidney for analysis – a much more direct way to analyse podocyte changes in the disease process compared to using isolated whole glomeruli. We used Western blot analysis of podocytes directly isolated from diabetic kidneys to quantify the increase in Smad4 protein levels, the decrease in synaptopodin protein levels, and the increase in ATPIF1 and PKM2 protein levels (see Fig 1C-E, Fig 2M-O, Fig 5A-B). These key findings could not be achieved by analysing isolated glomeruli. Furthermore, podocyte loss, decreased synaptopodin expression by podocytes and increased glomerular collagen IV deposition were quantified using immunohistopathology. Thus, we have provided a detailed and quantitative analysis of the key endpoints in experimental diabetic kidney disease.

3rd Editorial Decision 16 October 2019

Thank you for contacting us regarding the recent decision taken on your manuscript and providing your response to the referee concerns. Having seen that you and referee #1 are in disagreement regarding the validity of the model used, I have solicited additional expert opinion, which is copied below. The expert noted that HFD/STZ is a reasonable animal model of T2D, as the combination of the two accelerates emergence of the phenotype. Also having noted that referee #2 finds the model relevant, we decided to proceed with the publication of your manuscript, pending satisfactory minor revision. Before I can accept the manuscript, I need you to address the below editorial concerns.

- Please address the remaining minor concerns of the referee #2.
- We find the organization of figure 2 a bit confusing, please rearrange the panels.e.g. magnification of panel A is presented in C, which are intercalated by panel B.
- Figure 2, suppl. figure 1E and suppl. figure 3 are currently missing error bars.
- Moreover, the panels of figure 2 and suppl. figure 2 are not listed alphabetically in their respective legends.
- The suppl. figures should be renamed to expanded view (EV) figures. The callouts in the text should be updated accordingly.
- The callout for dataset should be corrected to Dataset EV1.
- The main figures should be uploaded as separate files.
- We have noted some ambiguities in the author contributions section. Namely, There are 2 WCs, therefore three letters should be used to differentiate them. There is a YX, does it refer to Xueqing Yu who is already listed as XY?

- Please fill out and include an author checklist as listed in out online guidelines (https://www.embopress.org/page/journal/14693178/authorguide)
- We realized that neither of the corresponding authors has linked their ORCiD accounts. As of January 2016, new EMBO Press policy asks for corresponding authors to link to their ORCID iDs. You can read about the change under "Authorship Guidelines" in the Guide to Authors here: https://www.embopress.org/page/journal/14693178/authorguide

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Advisor's comments:

I went through the rebuttal and original questions of the reviewers. Overall I think that the authors addressed the questions pretty well. Concerning the animal model, the HFD/STZ is a reasonable animal model of T2D. Indeed, it « accelerates » the phenotype, meaning that reaches the late stage of diabetes in a shorter time. Complications of diabetes are difficult to see in HFD fed mice, except if mice are treated with a low dose of STZ. There are increasing studies using this model. I would therefore consider that the results presented using this mouse model are relevant.

1st Revision - authors' response 31 October 2019

Referee #2:

Minor concerns

1) Please specify what "DN" and "DM" represents. The authors used both abbreviations in the figures, but they were never defined in the legends or in the text.

We have specified what "DN" and "DM" represents and defined them in the legends or in the text in the revised manuscript.

2) Figure 2D-F are of low quality and pixelated.

Figure 2D-F (now in Figure 3A-P) are of higher quality and pixelated in the revised manuscript.

3) Please state clearly the sample size when present a statistical analysis.

The sample size is clearly stated in the revised manuscript.

4) The LNA knockdown study in figure 1 to a large degree is to confirm the conditional KO of Smad4. I don't think this is of critical importance. It may be placed in the supplement to the paper. It feels unnatural to start the paper with this set of data.

We consider to leave the LNA-antisense as Figure 1. It is logical to start with the more general finding and then move to the more specific mechanism. It is also an important finding which should be in the main paper.

Accepted 18 November 2019

Thank you for submitting your revised manuscript. I have now taken a look at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jinhua Li/Xueqing Yu
Journal Submitted to: EMBO Report
Manuscript Number: EMBOR-2019-48781V3

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We routinely use groups of 6 to 8 mice in the T2D model. A group of 6 mice is sufficient for 90% power to detect a 40% difference in mean values with a 20% SD (or a 35% difference in means at 80% power).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We consider 35 to 40% changes to be biologically important in animal studies. Therefore, we used groups of 6 to 8 mice.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were excluded if they met criteria for euthanasia (10% loss of body weight, poor condition based on poor coat/isolated behaviour/lack of responsiveness).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	For the LNA treatment studies, mice were stratified based on litter, then randomised to the two treatments. For studies of different Smad4 genotypes, each litter were randomised to either high fat diet or normal diet.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were stratified based on litter, then randomized over the different treatments.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes. Mice were stratified based on litter, then randomized over the different treatments.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, the data meet the assumptions of the tests.
Is there an estimate of variation within each group of data?	Yes, we have inserted the estimate of variation within each group of data.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	rabbit anti-Tom20 conjugated with Alexa Fluor 647 (Abcam, UK, Cat. No.ab209606), guinea pig anti synaptopodin antibody (Synaptic Systems, GmbH, Goettingen, Germany, Cat. No.163004), goat anti-guinea pig Alexa Fluor 488 (Invitrogen, Mount Waverley, VIC, Australia, Cat. No. A18777), goat anti-Collagen IV (SouthernBiotech, Birmingham, AL, Cat. No. 1340-01), rabbit anti-fibronectin (Abcam, Cat. No. ab23751), donkey anti-goat Alexa Fluor 488 (Invitrogen, Cat. No.A11055), goat anti-rabbit Alexa Fluor 488 (Invitrogen, Cat. No. A11008), rabbit anti-Smad4 (Cell Signal Technology, Cat. No. 46535), mouse control IgG1 (Cell Signal Technology, Cat. No. 5415) or rabbit control IgG (Cell Signaling Technology, Cat. No. 3900),rabbit anti-synaptopodin (Synaptic Systems, Cat. No. 163002), anti-nephrin (Abcam, Cat. No. ab58968), anti-PKM2 (Cell Signal Technology, Cat. No. 4053), anti-ATPIF1 (Abcam, Cat. No. ab110277), MPC-1 (Cell Signal Technology, Cat. No.14462), rabbit anti-Smad4 (Cell Signal Technology, Cat. No. 46535), goat anti-rabbit (Sigma-Aldrich, Cat. No. 12348), goat anti-mouse antibody conjugated with HRP (Sigma-Aldrich, Cat. No. 12349),mouse anti-α-tubulin antibody conjugated with HRP (Cell Signal Technology, Cat. No. 9099), mouse anti-GAPDH antibody conjugated with HRP (Cell Signal Technology, Cat. No. 51332),rabbit anti-nephrin conjugated with Alexa Fluor 488 fluorophore (Bioss-USA, Woburn, MA, Cat. No. bs-10233R-A488).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Mouse podocyte cell line was a gift from Professor Jeffrey B. Kopp, NIH, Bethesda, MD). Mycoplasma contamination was not tested recently, we did not report here.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Breeding pairs of eNOS-/- mice, Smad4fl/fl mice, tamoxifen-inducible Tg(CAG-cre/ Esr1)5Amc/J mice (ER-Cre), and 2.5P-Cre;Tg(NPHS2-cre)1Lbh mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at Monash Animal Services. Male mice were used only in the study due to male mice being more sensitive to streptozotocin-induced islet cell damage.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were approved by the Monash University Animal Ethics Committee and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirmed compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Studies using human tissue were approved by the Human Ethics Committee of Monash Medical Centre.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Written informed consent was obtained from the patients (to use biopsy tissue excess to that required for diagnosis for scientific research purposes) and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.