

Placental TRPV2 supports trophoblast function and normal fetal development.

Corresponding Author: Professor Katrien De Clercq

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, the authors convincingly showed that Trpv2 KO mutation disrupted placental morphology, leading to fetal growth restriction and lethality, attributed to its non-redundant function in extraembryonic trophoblast cells rather than in the embryonic tissues. Using CRISPR/Cas9-mediated Trpv2 deletion in mouse trophoblast (TS) stem cells, we demonstrate that TRPV2 is critical for the differentiation of Syncytiotrophoblast layer II (SynTII) cells, as confirmed by the growth restriction phenotype observed in SynTII-specific Trpv2 knockout models. Additionally, in human placentas, TRPV2 expression was reduced in cases of early-onset fetal growth restriction compared to normal pregnancies. These findings highlight the indispensable role of TRPV2 in placental development and its direct impact on fetal outcomes. Overall, this is an excellent study addressing crucial biomedical questions. The manuscript is well-written, and experimental data support the main conclusions. I have only a few critical points to address.

Major concerns:

Functional assessment of mTS cells was conducted using the Ca²⁺ imaging approach, which is limited in sensitivity, especially in the evaluation of endogenous TRP channels. In light of the strong background of this laboratory in the electrophysiological analysis of TRP channels, including TRPV2, this is an unexpected choice. The study will be significantly improved if whole-cell TRPV2 currents are measured in WT cells but not in the cells with the KO mutation. This issue is even more critical for assessing the TRPV2 channel in human hTS cells, conducted without KO controls, especially after differentiation towards ST cells when TRPV2 was found to be up-regulated. Furthermore, Figure 5E provides too few details. Can Ca²⁺ traces be presented like in Figure 5D? What was the effect of THC on Ca²⁺ levels in human cells?

Minor points:

Introduction

Lines 49-50: Please refer to publications supporting the statement 'While abnormal placental growth or function is a main cause of FGR...'

Line 71: 'However, a role for TRPV2 in murine or human placenta has never been described.' This is a confusing notion. Upstream, the author referred to their original papers that focused on the function of TRPV2 in the placenta. Please clarify.

Results

Lines 104-107: Remarkable, however, was the significant Trpv2 expression in the mouse placenta compared to other organs in the embryo or adult (Supplementary Fig. 2b), and the identification of Trpv2 as specifically enhanced within the mouse placenta, similar to other placental specific genes, including Phlda2 (Supplementary Fig. 2c). This sentence is unclear. Please edit.

Lines 199-200: Stemness of mTSC was unaffected in Trpv2-null mutants, evidenced by similar expression of Eomes, Elf5

and Cdx2. To support this conclusion, please report how long Trpv2 KO mTS cells could be maintained in culture (i.e., number of passages).

Lines 202-206: For in vitro differentiation of mTS cells towards SynTII cells, it would be more informative if the author provided images of differentiated WT and Trpv2 KO cells.

The mortality rate was not found in Trpv2 SynTII KO animals, unlike KO, which Sox2cre induced. The author should discuss this mismatch in more detail and outline possible reasons.

Discussion

It will be interesting to correlate the placental phenotype of Trpv2 KO mice with other mouse strains carrying mutations in TRP channels. Is the Trpv2 KO phenotype unique or just more/less robust?

Reviewer #2

(Remarks to the Author)

This manuscript by De Clerq and colleagues describes a conditional mouse mutant analysis of the Ca²⁺ channel gene Trpv2. By data mining, the authors find that Trpv2 is highly expressed in the placenta compared to other tissues, and that it is specifically expressed in trophoblast giant cells, in the junctional zone and in SynTII precursors and SynTII cells of the murine labyrinth, as corroborated experimentally. Constitutive KO of Trpv2 leads to late gestational growth retardation and increased fetal mortality around term, or immediately post partum. Placentas are also growth retarded and exhibit an unusual phenotype in which certain areas are devoid of fetal capillaries and SynTII cells, whereas the normally juxtaposed SynTI cells are still present in these areas. Embryo-specific deletion does not rescue the phenotype, pointing to a vital role of Trpv2 in the placental trophoblast compartment. JZ-specific deletion does not induce a phenotype, but SynTII-specific deletion exhibits a phenotype akin to the constitutive KO, albeit considerably milder. Finally, the authors also show that placentas from human pregnancies affected by early-onset FGR exhibit lower Trpv2 expression levels.

Overall, this study establishes an essential function of Trpv2 in the placenta, and specifically in the establishment of the transport surface of the placenta, in mice and perhaps also in humans.

The experiments are carefully conducted, the figures are very pretty and, for what they are, the data are convincing. The corroboration of cell type-specific activity of the conditional CRE lines is much-appreciated evidence of due diligence, although this has been published before. The authors have also taken full advantage of data mining and meta-analysis approaches of several published sequencing datasets in a meaningful way. However, in light of an assessment for Nature Communications, the overall impact and novelty of the study is limited. The finding that late-gestational growth retardation and fetal lethality is often due to placental insufficiency is not new, and there are numerous genes and mouse models for which this has been shown. The phenotype of the mutant labyrinth is interesting, but the precise cell lineage relationships and the identity of these unusual Trpv2-positive precursor (?) cells has not been followed up to the point of fully resolving what these cells are. For these reasons, despite the comprehensive nature of this analysis, the impression is that the scope of the study falls short of the remit for this particular journal.

Additional comments:

It is highly unlikely that all the Synb- Trpv2+ cells are SynTII precursors. Visually, the overlap of the ISH signal with Slc16a1 is much higher than with Synb, perhaps locating to different aspects of the same cells. Although the double-ISH stainings are extremely nice, the visualization of mRNA leaves room for ambiguity. Attempts should be made to better resolve cell type-specific expression in a clearer way, perhaps by ISH combined with IF. This might help establish the cell lineage relationships. Ideally, lineage tracing of Trpv2-expressing cells would be required to resolve the question of what cells /progenitors precisely express this gene. The suspicion around the SynTII assurance is corroborated by data in Suppl. Fig. 14b that show no response in Trpv2 expression to CHIR, hence a non-SynTII-associated expression pattern. How do other SynT precursor markers behave in the presence of CHIR? (Also, Suppl. Fig. 6c is in a different colour tone.)

Little is made of the RNA-seq data beyond confirmation of down-regulation of SynTII genes. Yet even at E14.5 before the onset of FGR, the placental transcriptome is highly enriched for GO terms related to immune cell activation. What precisely are these much more prominently de-regulated, upregulated genes (Fig. 3c, Suppl. Fig. 12a)? Do they contribute to the phenotype? How does that translate to particular cell type(s) in the placenta that are proportionately more abundant (or alternatively, what cell types highly over-express these genes), and what relationship is there between these genes and TRPV2 function as a Ca²⁺ channel?

What happens to Trpv2 mutant pups when wildtype and heterozygous littermates are removed? Does the lack of competition allow Trpv2^{-/-} pups to survive? Previous data have reported reduced viability but seemingly did obtain some surviving KO animals. Along these lines, are epiblast-specific KO mice fully viable, i.e. have they been assessed beyond term?

The lectin/cytokeratin stainings are unclear, the blue is faint and at times 'washed out', in particular in Fig. 4. Please replace with better examples or other staining types. Also please label what is stained in which colour.

What is in the interspaces of SynTI-only areas? Is maternal blood present? Presumably no fetal blood? What other cells occupy this space?

What time point was designated “term” in the mouse? E19.5? Were the females in labour?

Given the relatively little expression of TRPV2 in ST of human placentas but prominent staining in villous mesenchymal cells, the down-regulation of TRPV2 in FGR placentas may originate from non-trophoblast cell types. Have the authors assessed histological sections of these placentas for TRPV2 expression to determine in which cell types it is depleted?

Minor comments:

Figures:

Figure 2 is very low resolution in the PDF.

Fig. 2h: there is considerable variability in the labyrinth phenotype, sometimes almost following a bimodal pattern. Is there a genetic explanation for this (e.g., *Trpv2* imprinting or allelic bias in the heterozygous uterus)?

Fig. 4d, overview image: Staining is of poor quality.

UMAP plots: It would be helpful to label pertinent branches for easier identification, the colour scheme is very hard to make out.

Suppl. Fig. 1c: The Sox2-Cre genotype is not relevant here.

Suppl. Fig. 2c: Genes and rows are mis-aligned

Supp. Fig. 7: typo in figure element annotation

Fig. 1 and Suppl. Fig. 3a: Nice diagrams, but the embryos and placentas are a little wonky, which in part might be a PDF conversion issue (some lines sticking out, the placental drawing could be improved). What is a (F)/+/- genotype? Also, what precisely is meant by the sentence in the legend, “Occasionally (8-12%), constitutive deletion was observed and represents full *Trpv2*^{-/-} mice, that were not assessed” – When was this observed (i.e. in which breeding strategy), how was it determined and how would constitutive deletion come about?

Text:

Please correct C57BL/6 mouse strain nomenclature in a few instances.

Line 92: Fetal growth ‘velocity’ is a strange term, presumably fetal growth rates or weight gain is meant here.

Line 113: “Reconstituting TRPV2 function only in the extraembryonic tissues” – this is misleading, as the gene was not reconstituted in trophoblast, instead it was specifically deleted from the epiblast-derived tissues

Lines 156, 159: What do the authors mean by ‘cellular dysplasia’ or ‘labyrinth dysplasia’?

Line 172: Fig. 3a should presumably be Fig. 3b

Reviewer #3

(Remarks to the Author)

Key results

The authors performed tissue specific and total KO of TRPV2 in mouse and found that it is essential in the placenta but not in the embryo – resulting in FGR and embryonic lethality in the total KO and with no effect in embryo-specific KO.

CRISPR-cas9 mediated deletion of *Trpv2* in mTSCs resulted in a reduction of SynT-II marker expression, which was confirmed using SynT-II specific KO in mouse that partially replicated the FGR phenotype.

The authors also investigated the expression of *Trpv2* in human placentas with FGR and preterm birth with FGR and found that *Trpv2* expression was significantly decreased in placentas with preterm birth and FGR but not term FGR (see further comments relating to this below).

Validity

The results are largely accurately and carefully described in the text, however the conclusions drawn are often exaggerated and fail to account for experimental limitations. This is particularly in relation to the human placenta data presented in the manuscript and to the claim that *Trpv2* is “indispensable” for SynT-II differentiation.

This manuscript requires significant revision and further experiments to strengthen the evidence presented.

Significance

The key role of the placenta in normal development is frequently overlooked. Therefore, this study which investigates the role of a *Trpv2* in specific components of the mouse placenta is important. Defining the mechanisms which underly placental development and function, and how these may contribute to disease is an important area of work. This manuscript describes sophisticated genetic manipulation which allows the role of *Trpv2* to be studied in the different placental compartments. This adds important new information to our understanding of placental function.

Data and methodology

Mouse knock-outs:

The knock-out of *Trpv2* in the whole mouse or specific tissues is a well-designed and logical strategy. However, I have 2 significant reservations about aspects of this work.

i) In the previous cited paper describing the generation and analysis of *Trpv2* knock-out mice there was a very notable background-dependent effect on the phenotype. Specifically, in a BL/6 background (as in the study reported here) there was a very high rate of embryonic death close to term. However, in a mixed (BL/6 x 129S) cross, survival was markedly improved

(although not completely normal). Thus, the strong claims made here that TRPV2 is “indispensable” (line 72 and elsewhere) is not true in a mixed genetic background. Such background-dependent effects are not uncommon in gm mice but the conclusions need to reflect this.

ii) The logical progression from complete knock-out and the rescue in epiblast specific ablation points very strongly to a placental (indeed a trophoctoderm lineage) dependent phenotype. This is solid. The lack of an obvious effect when the loss is confined to the ectoplacental cone (Tpbpa+) lineage points strongly to the placental labyrinthine zone. However, when the loss is confined to Gcm1+ cells the effect is considerably weaker – there is no perinatal death and only an embryonic growth defect. This suggests that placental Gcm1-ve cells contribute to the phenotype in some way. This is not discussed and it needs to be.

mTSC data:

The authors used CRISPR-cas9 mediated deletion of Trpv2 in mTSCs which is well described. KO of Trpv2 resulted in a reduction in SynT-II marker gene expression as measured by qPCR, however the data are rather noisy. In Fig 3h and S14c the differences observed are significant only for one marker – Gcm1. This is not sufficient to claim that SynT-II differentiation is impaired in the KO mTSCs.

A SynT-I fusion defect was observed in the Trpv2 KO in mouse placental labyrinth IHC in Fig 3b. However, Trpv2 KO had no effect on the SynT-I markers measured in mTSCs or in mouse labyrinth. The authors should explain these results.

The observation that Gcm1 is consistently decreased with Trpv2 KO in different experimental modalities highlights that Trpv2 may exert some upstream control over Gcm1 expression. The authors should comment on this, especially considering the crucial role Gcm1 plays initiating SynT-II differentiation.

Use of publicly available datasets:

The authors have efficiently utilised publicly available datasets to strengthen their claims and direct further experiments.

Human data:

The localisation of TRPV2 mRNA differ in the human and mouse placenta – in the mouse it is predominantly in the syncytium but in the human it is mainly in the villous core. One notable exception is in the boxed region (1) in Fig 5a in which the CGA negative cells which appear to syncytium are TRPV2 +ve. This is not a big problem but it warrants some comment in the discussion

L 235, the statement that the syncytium is “mainly responsible for nutrient transfer” needs to be removed – this layer is also a major site of hormone production - as the authors themselves show in Fig 5a (CGA mRNA is extremely abundant).

L 252 TRPV2 a “defining feature”, this is a very strong statement and needs to be toned down.

L 258 The claims about relationship between placental TRPV2 mRNA and human fetal growth restriction are weak. A major challenge when investigating the relationship between placental mRNA, (or protein etc) and pathological conditions is that such analyses are confounded by gestational age. I.e, many important pathologies (pre-eclampsia, fetal growth restriction etc) lead to early delivery so comparing such samples with samples collected at term is invalid. Samples need to be matched for gestational age but samples collected pre-term are by definition not normal. This is an insurmountable challenge in placental research. Although the authors clearly acknowledge this (line 306) their conclusions ignore this and need to be more cautious. Specifically, there is no difference in TRPV2 mRNA when comparing the term FGR cases and controls.

Hence the data from the pre-term FGR cases should not be compared with the term AGA samples. This leaves the 4 term early-onset FGR cases and 4 is a very small sample size. The details of some maternal characteristics (age, parity, BMI and mode of delivery) and fetal sex, indicate considerable variation. Just because there are no “significant” differences in these indexes it does not mean the groups are uniform. This is particularly true when there are only 4 subjects in one group. In addition, no neonatal outcomes are described. Although the participants were recruited as part of the EVERREST trial, it is not clear which centre collected these samples. All this means that any conclusions drawn from these 4 placentas must be treated with considerable caution and they are of necessity, weak. However, the authors’ claims in the title and abstract are insufficiently cautious.

Suggested improvements

Quantifying cell fusion and synT-I and II markers (using IHC) in the differentiating WT and KO mTSCs would significantly strengthen the claim of impaired SynT-II differentiation. Additionally, it would allow investigation of whether synT-I fusion defects are also present in mTSCs as described in fc-void areas in the mouse labyrinth.

Minor comments:

The text is too small to read in Figure S2

Stereology – the units used to report this are confusing. It should either be a “fraction” or if corrected for tissue volume, then a volume (mm³)

Line 214 Fig S15b – note Trpv2 is not “highly expressed” in the differentiating

L 217 Fig 4 – legend and labelling have errors, particularly 4b-d and why are the fetal weights shown as averages and placental weights as individual data points?

L221 – It is impossible to see cell morphology at the magnification shown.

L 234 the images in Fig 5a are too small and it is not possible to see the stained cells in the villus core. Similarly, Figs. S16a and b are much too small.

L 365 “stillborn” – are the authors sure these animals were not born live and died shortly after birth? – I suspect not so please reword.

Line 419-420 – “antibody” was not detected with the streptavidin-HRP – it was the biotinylated lectin.

L774 Fig 3g has some rendering issues, a colour key is not where it should be and extra lines are present.

L789 Fig 4a – adding a column with the expected mendelian ratios would be very helpful for reading the figure.

L811 – “independent differentiation protocols” and “suggestive” need rephrasing

L817 – “Turkey” should be Tukey

Clarity and context

The information provided about how public datasets were re-analysed is insufficient to replicate the analysis – adding this to the methods would be helpful

The level of detail provided on the generation of the different KO mouse lines is very different between the different lines – this should be expanded.

References

Line 60-62: There is a reference missing for the role of Trpv2 channels in the cell.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all of my critical points in the revised manuscript.

Reviewer #2

(Remarks to the Author)

This revised study by De Clerq and colleagues investigates the role of the Ca²⁺ channel TRPV2. Using a series of constitutive and conditional knockouts, they show that the fetal growth retardation and propensity to perinatal lethality are driven by a placental dysfunction phenotype that is introduced by Trpv2 deletion. Specifically, they show a defect in syncytiotrophoblast layer II formation in Trpv2 mutants in quite some exquisite detail. In line with a primary function of TRPV2 in this particular cell type, a SynT-II-specific ablation of Trpv2 recapitulates significant aspects of the phenotype. They also explore the function of TRPV2 in human trophoblast cells and conclude that it may play similar roles also in human placentation, even if this conclusion remains somewhat speculative.

The overall message of the manuscript has not changed from the first submission, but the level of detail in which the phenotype has been assessed is substantially bolstered. The RNAscope and MERfish-based spatial transcriptomics data are impressive. In particular, the subsetting into normal and abnormal placental regions in the spatial transcriptomics experiments allows for a highly detailed level of resolution. Collectively, this manuscript is now far better aligned with the target journal, and the authors should be commended for the efforts they made in this revision to address the various points raised by the reviewers.

Some remaining, minor points should be fixed:

Introduction:

Line 51, normal functioning placentation – please re-word

Line 52/53: Please refrain from referring to syncytiotrophoblast cells or even endothelial cells as being “invasive”. In the context of placental development, this is a very confusing terminology that has nothing to do with the trophoblast invasion associated with spiral artery remodelling, which is attributed to entirely different cell types both in mice and in humans. Instead, please describe this as “invaginating” fetal blood vessels into the chorionic trophoblast cell layer, in line with the morphological movements (as opposed to single cells invading into a foreign tissue). Equally in line 55/56, I suggest to delete the “characterized by their invasive properties” part of the sentence.

Line 63, we don’t know whether TRP channels “excel” in this process, rephrase to “function” or similar

Line 66 and also Results, it remains unclear what proportion of Trpv2 mutants survives. Please specify these numbers (1) from previous KO studies and (2) in the current study.

Results:

The description of the genetics around the Sox2-cre transgene are very confusing, what is transmitted is the CRE protein via the oocyte, which results in the excision of floxed alleles even in the absence of the Sox2Cre transgene itself. Please simplify to this end. The phrase “full body heterozygous deficient animals” is an oxymoron, “heterozygous” will suffice. Also in Suppl Fig. 3, top panel, simplify this to a +/- intercross. The Sox2-Cre is irrelevant here. As mentioned in the previous review, for a reader with less knowledge about this particular Cre line, this will be confusing. In this type of cross, it does not matter which Cre deleter was used to accomplish the mutant allele.

Related to that, lines 116-117, just state that Trpv2 was deleted from all epiblast derivatives using the Sox2-cre transgene, including the fetal endothelial cells of the placenta. Currently, the sentence implies that endothelial cells are Sox2-positive.

If only 25% of Trpv2^{-/-} fetuses die (i.e. one quarter of the 25% by Mendelian ratios, so that would be 6.25% dying and 18.75% surviving), how does that result in 2% of born offspring mentioned a few lines previously? These numbers are hard to follow, please describe these numbers more clearly.

Line 100 – Concomitantly

Supp 2b, there seems to be a gene name missing for the top row (i.e., it is presumably Tpbpa, third from the top, that is so highly enriched in placenta)

The Trpv2 enrichment in this heatmap is not very clear, it looks almost as abundant in bone marrow, for example. Fig. S2a is clearer.

Fig. S8b, e, please label the trajectories on the UMAP, the colour-code is indiscernible

Fig. S10g, what happened to the mutant placenta photo? What are all the background pixels? Please provide a better photo or histological specimen.

Line 185, “given the absence of Tpbpa expression”

Discussion

Even if Word counts may be limited, please allude to a potential mechanism of how a Ca²⁺ channel could affect determination and differentiation of one particular trophoblast cell type. Are PIP2 augmentation and actin depolymerization (or other processes associated with Trpv2) specifically important for SynT-II formation.?

Materials and Methods

Related to Fig. 4, why is the empty vector control called “scr”? The methods don’t state that a random 20nt sequence was used as control. Please re-label Fig. 4 accordingly (“vec”, “EV”, “Ctrl” or similar). Were the controls also single cell-derived clones?

Reviewer #3

(Remarks to the Author)

See the attached document.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have addressed all remaining minor concerns.

Reviewer #3

(Remarks to the Author)

All my previous concerns have now been address satisfactorily.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

REVIEWER COMMENTS

Reviewer #1:

In this study, the authors convincingly showed that Trpv2 KO mutation disrupted placental morphology, leading to fetal growth restriction and lethality, attributed to its non-redundant function in extraembryonic trophoblast cells rather than in the embryonic tissues. Using CRISPR/Cas9-mediated Trpv2 deletion in mouse trophoblast (TS) stem cells, we demonstrate that TRPV2 is critical for the differentiation of Syncytiotrophoblast layer II (SynTII) cells, as confirmed by the growth restriction phenotype observed in SynTII-specific Trpv2 knockout models. Additionally, in human placentas, TRPV2 expression was reduced in cases of early-onset fetal growth restriction compared to normal pregnancies. These findings highlight the role of TRPV2 in placental development and its direct impact on fetal outcomes. Overall, this is an excellent study addressing crucial biomedical questions. The manuscript is well-written, and experimental data support the main conclusions. I have only a few critical points to address.

We thank the reviewer for the overall positive appraisal of our manuscript.

Major concerns:

Functional assessment of mTS cells was conducted using the Ca²⁺ imaging approach, which is limited in sensitivity, especially in the evaluation of endogenous TRP channels. In light of the strong background of this laboratory in the electrophysiological analysis of TRP channels, including TRPV2, this is an unexpected choice. The study will be significantly improved if whole-cell TRPV2 currents are measured in WT cells but not in the cells with the KO mutation. This issue is even more critical for assessing the TRPV2 channel in human hTS cells, conducted without KO controls, especially after differentiation towards ST cells when TRPV2 was found to be up-regulated. Furthermore, Figure 5E provides too few details. Can Ca²⁺ traces be presented like in Figure 5D? What was the effect of THC on Ca²⁺ levels in human cells?

We thank the reviewer for highlighting the importance of direct electrophysiological validation. In response, we have performed whole-cell patch-clamp recordings in both mouse and human trophoblast models. These data confirm functional TRPV2 channel activity in human and mouse trophoblast cells and support the conclusions drawn from calcium imaging. We included:

- Whole-cell TRPV2 currents were measured in mTSC WT and KO cells on D2 of differentiation. The results were included in Supplementary Figure 17.
- Whole-cell TRPV2 currents were measured in ST D1, and the results were added in a new Supplementary Figure 20.
- Figure 5e, which is now Figure 6e in the revised manuscript, was adapted to contain calcium traces and THC responders, and was further explained in text/methods and figure legend.
- Live imaging of calcium signals revealed functional expression of TRPV2 in hTSC and in early STs. Interestingly, while TRPV2 mRNA levels increased during ST differentiation, functional activation by THC became less robust at later stages. This may reflect higher basal intracellular calcium levels or altered channel availability, rather than absence of TRPV2 expression. These possibilities are currently under investigation.

Minor points:

Introduction

- Lines 49-50: Please refer to publications supporting the statement 'While abnormal placental growth or function is a main cause of FGR...'

This statement was supported by 2 recent reviews reporting on the placental causes of FGR.

- Line 71: 'However, a role for TRPV2 in murine or human placenta has never been described.' This is a confusing notion. Upstream, the author referred to their original papers that focused on the function of TRPV2 in the placenta. Please clarify.

While we previously reported prominent *Trpv2* expression in the mouse placenta, its functional role and the consequences of *Trpv2* deletion for placental development had not been investigated. The text was revised to clarify this distinction:

"Nevertheless, its functional role in early placental development has not been addressed."

Results

- Lines 104-107: Remarkable, however, was the significant *Trpv2* expression in the mouse placenta compared to other organs in the embryo or adult (Supplementary Fig. 2b), and the identification of *Trpv2* as specifically enhanced within the mouse placenta, similar to other placental specific genes, including *Phlda2* (Supplementary Fig. 2c). This sentence is unclear. Please edit.

We apologize for sloppy wording and have rephrased accordingly:

*"Remarkable, however, was the significant *Trpv2* expression in the mouse placenta compared to other organs in the embryo or adult (Supplementary Fig. 2a). Moreover, *Trpv2* was identified as specifically enhanced within the mouse placenta, meaning that its expression in the placenta is at least five-fold higher compared to the average levels in all other tissues (Supplementary Fig. 2b)."*

- Lines 199-200: Stemness of mTSC was unaffected in *Trpv2*-null mutants, evidenced by similar expression of *Eomes*, *Elf5* and *Cdx2*. To support this conclusion, please report how long *Trpv2* KO mTS cells could be maintained in culture (i.e., number of passages).

We thank the reviewer for this suggestion and have added a sentence for clarification. Both WT and KO mTSC were kept in culture for at least 10 passages. Moreover, a recent study in hTSC also excluded TRPV2 as an essential gene for stemness¹. Thus, *Trpv2* deletion does not seem to compromise long-term self-renewal capacity.

*"Stemness of mTSC was unaffected in *Trpv2*-null mutants as they appeared to self-renew and expand for over 10 passages, and evidenced by similar expression of *Eomes*, *Elf5* and *Cdx2*."*

- Lines 202-206: For in vitro differentiation of mTS cells towards SynTII cells, it would be more informative if the author provided images of differentiated WT and *Trpv2* KO cells.

We agree that visualization of differentiated cells would in principle provide additional context for the in vitro experiments. However, we did not include representative images of

SynTII-directed differentiation, as morphological assessment in this system is highly variable and does not reliably reflect lineage specification. Under these conditions, differentiation outcomes are heterogeneous and not tightly controlled, such that representative images may not accurately capture the underlying biology. Instead, we focused on quantitative transcriptional readouts, including RNA sequencing and gene set-based analyses, which provide a more robust and reproducible assessment of SynTII differentiation.

We have, however, included images of mTSCs under stem cell conditions (Figure 4a) to illustrate overall cell morphology. Moreover, in response to reviewer #3, we included in the rebuttal an image of SynTII differentiation. If the reviewers and editors consider it essential, we would be happy to include the images of differentiated cultures with the appropriate caveats.

- The mortality rate was not found in *Trpv2* SynTII KO animals, unlike KO, which *Sox2cre* induced. The author should discuss this mismatch in more detail and outline possible reasons.

We thank the reviewer for the opportunity to clarify this important point, which could not be addressed in detail in the original submission due to space limitations.

First, the phenotype of *Trpv2* knockout mice is strongly strain dependent (see Discussion). *Gcm1*-Cre mice were originally generated on a 129S background, and *Trpv2* knockout viability is known to be markedly improved on this background. Indeed, as originally reported by Park et al. (2011)² and confirmed in our own breeding experience, *Trpv2*^{-/-} mice on a C57BL/6 background show <2% survival, whereas backcrossing to 129S markedly improves viability. Thus, background effects likely contribute to the absence of lethality in the SynTII-specific knockout.

Second, the *Gcm1*-Cre deleter strain exhibits sporadic expression of *Gcm1* in male germ cells (Supplementary Fig. 19c), which occasionally results in ubiquitous deletion of *Trpv2* throughout the entire conceptus (*Gcm1*-Cre^{wt/tg}; *Trpv2*^{-/-}, hereafter referred to as *Gcm1*.*Trpv2*^{-/-}). This allows direct comparison within the same litter between complete *Trpv2* knockouts generated via the *Gcm1*-Cre line and SynTII-restricted *Trpv2* knockouts (*Gcm1*-Cre^{wt/tg}; *Trpv2*^{F/-}). Notably, the complete *Gcm1*.*Trpv2*^{-/-} animals did not exhibit late embryonic lethality, in contrast to global *Trpv2* knockouts generated using *Sox2*-Cre, indicating that lethality is not an inevitable outcome of complete *Trpv2* deletion.

Third, *Trpv2* and *Gcm1* are not exclusively co-expressed. While *Gcm1* expression is restricted to SynTII cells, not all SynTII cells express *Gcm1*³, and *Trpv2* is additionally expressed in *Gcm1*-negative cells (Figure 2). Analysis of published snRNA-seq datasets⁴ demonstrates that co-expression of *Trpv2* and *Gcm1* is lower in mononuclear SynTII precursors than in fully differentiated SynTII cells, indicating incomplete overlap between the two lineages.

Together, incomplete lineage targeting combined with strain-dependent resilience provides a parsimonious explanation for the milder phenotype and lack of fetal lethality observed in *Trpv2*^{SynTII-/-} mice. Accordingly, we now concisely discuss these factors in the revised manuscript:

“This difference in phenotypic severity likely reflects strain-dependent differences in Trpv2 knockout severity or the broader expression of Trpv2 across trophoblast differentiation trajectories, including precursor

populations and additional lineages such as S-TGCs, suggesting that TRPV2 contributes to placental development at multiple stages of trophoblast differentiation.”

Discussion

- It will be interesting to correlate the placental phenotype of *Trpv2* KO mice with other mouse strains carrying mutations in TRP channels. Is the *Trpv2* KO phenotype unique or just more/less robust?

We appreciate the reviewer’s suggestion to contextualize the *Trpv2* phenotype relative to other TRP channels implicated in placental or reproductive biology. Based on our previous systematic analysis of TRP channel expression across murine placental development (De Clercq et al., 2021)⁵, only a limited subset of TRP channels are expressed within trophoblast lineages, most prominently TRPV2, TRPM6, and TRPM7.

- Deletion of *Trpm7* results in embryonic lethality prior to E7, including in epiblast-specific knockouts, indicating an essential embryonic function that precludes meaningful evaluation of placental morphogenesis, despite expression of *Trpm7* in trophoblast stem cells.^{6,7}
- *Trpm6* knockout mice exhibit lethality between E9.5 and E14.5, largely attributed to systemic magnesium deficiency and neural tube defects. Although epiblast-specific *Trpm6* knockouts are viable, placental morphology has not been examined in global knockouts, and impaired placental development per se has not been discussed.⁸
- Other TRPV family members (TRPV1, TRPV3, TRPV4) show minimal or absent expression in trophoblast lineages during mouse placental development, and their deletion has not been associated with placental insufficiency or fetal growth restriction.
- TRPV6, while highly expressed in placental tissues, is largely restricted to the intraplacental yolk sac and is not expressed in mouse trophoblast lineages.
- TRPC6 deficiency has been linked to placental defects, including impaired labyrinth vascularization and fetal growth restriction. However, this phenotype reflects altered syncytiotrophoblast function and vascular organization rather than defective trophoblast lineage emergence.

In contrast, *Trpv2* deletion disrupts the formation of syncytiotrophoblast layer II itself, indicating a role at the level of trophoblast lineage development. Collectively, these observations suggest that TRPV2 contributes to placental development through a mechanism distinct from other TRP channels studied to date. We have now incorporated this comparison into the Discussion with references as follows:

“Moreover, several TRP channels, including TRPM6 and TRPM7, have been implicated in early development and trophoblast biology³⁴⁻³⁶, but their roles are either masked by early embryonic lethality or primarily affect placental function rather than lineage formation. In contrast, TRPV2 appears to regulate syncytiotrophoblast II development itself, suggesting a distinct role in placental morphogenesis.”

Reviewer #2:

This manuscript by De Clercq and colleagues describes a conditional mouse mutant analysis of the Ca²⁺ channel gene *Trpv2*. By data mining, the authors find that *Trpv2* is highly expressed in the placenta compared to other tissues, and that it is specifically expressed in trophoblast giant cells, in the junctional zone and in SynTII precursors and SynTII cells of the murine labyrinth, as corroborated experimentally. Constitutive KO of *Trpv2* leads to late gestational growth retardation and increased fetal mortality around term, or immediately post partum. Placentas are also growth retarded and exhibit an unusual phenotype in which certain areas are devoid of fetal capillaries and SynTII cells, whereas the normally juxtaposed SynTI cells are still present in these areas. Embryo-specific deletion does not rescue the phenotype, pointing to a vital role of *Trpv2* in the placental trophoblast compartment. JZ-specific deletion does not induce a phenotype, but SynTII-specific deletion exhibits a phenotype akin to the constitutive KO, albeit considerably milder. Finally, the authors also show that placentas from human pregnancies affected by early-onset FGR exhibit lower *Trpv2* expression levels. Overall, this study establishes an essential function of *Trpv2* in the placenta, and specifically in the establishment of the transport surface of the placenta, in mice and perhaps also in humans.

The experiments are carefully conducted, the figures are very pretty and, for what they are, the data are convincing. The corroboration of cell type-specific activity of the conditional CRE lines is much-appreciated evidence of due diligence, although this has been published before. The authors have also taken full advantage of data mining and meta-analysis approaches of several published sequencing datasets in a meaningful way. However, in light of an assessment for Nature Communications, the overall impact and novelty of the study is limited. The finding that late-gestational growth retardation and fetal lethality is often due to placental insufficiency is not new, and there are numerous genes and mouse models for which this has been shown. The phenotype of the mutant labyrinth is interesting, but the precise cell lineage relationships and the identity of these unusual *Trpv2*-positive precursor (?) cells has not been followed up to the point of fully resolving what these cells are. For these reasons, despite the comprehensive nature of this analysis, the impression is that the scope of the study falls short of the remit for this particular journal.

We agree that more detailed characterization of the mutant labyrinth phenotype was required. To address this, we performed pseudobulk transcriptomic analysis of region-based MERscope spatial transcriptomics at E16.5 using a targeted gene panel enriched for trophoblast and vascular markers. Rather than relying on cell-level segmentation, which is challenging in multinucleated and syncytial trophoblast lineages, we analyzed spatially defined regions. This revealed that abnormal labyrinth areas in *Trpv2* deficient placentas are transcriptionally coherent and characterized by coordinated loss of mature SynTII programs together with selective persistence of alternative trophoblast states, along with global reduction of vascular and mural-associated programs. These data demonstrate that the mutant labyrinth phenotype reflects structured alteration of trophoblast differentiation and organization rather than undefined or damaged tissue. This was further discussed in the text and added in Figure 3 and Supplementary Figures 15-16. This distinguishes the present study from previously reported models in which reduced SynTII markers were observed but without resolution of the underlying cellular mechanism.

We further thank the reviewer for raising this important point regarding the identity of *Trpv2*-expressing cells that do not overlap with canonical SynTII markers in the early labyrinth. We agree that the original wording and presentation may have conveyed unnecessary ambiguity regarding the nature of these *Trpv2*⁻*Synb*⁻ populations. To directly

address this concern, we have substantially revised both the Results text, and Figure 2 and Supplementary Figures 5-9, and added new MERscope-based spatial transcriptomic analyses at E9.5, with the explicit aim of clarifying when and where *Trpv2* is expressed during early labyrinth morphogenesis, rather than framing these cells primarily by what lineage markers they lack.

We therefore explicitly introduce the MERscope approach in the Results and use it to provide spatial context for *Trpv2* expression at E9.5, a developmental window in which trophoblast lineages are still being established.

- First, rather than emphasizing the absence of *Synb* expression, we have reframed the Results to describe *Trpv2* expression across spatially distinct trophoblast domains within the early labyrinth. The revised text shows that *Trpv2* robustly overlaps with *Synb* in regions corresponding to differentiating SynTII cells but is also detected in adjacent regions where *Synb* expression is minimal and early labyrinth markers such as *Slc5a5* and *Gcm1* are present. Importantly, we no longer describe these domains as a discrete precursor compartment but instead present them as spatially heterogeneous regions within the same tissue plane, consistent with early labyrinth development.
- Second, to further resolve the identity of *Trpv2*⁺ populations outside overt SynTII domains, we integrated additional MERscope analyses of *Mfsd2a*. We show that *Trpv2* co-expression with *Mfsd2a* marks early labyrinth regions at E9.5, while at later stages (E16.5) *Mfsd2a* is restricted to mature S-TGCs and co-localizes with established S-TGC markers (*Cdh23*, *Lepr*). This temporal and spatial correspondence supports the interpretation that a subset of early *Trpv2*⁺ cells align with an S-TGC-biased differentiation trajectory, well before acquisition of terminal markers, rather than representing undefined or aberrant cell types.
- Finally, we now clearly state that *Trpv2* shows little systematic overlap with SynTI lineage markers (*Spata13*, *Tgfa*, *Atp2a3*, *Syna*) in early labyrinth stages.

In summary, we have completely revised the wording and figure structure to remove the perceived “mystery” surrounding *Trpv2*⁺*Synb*⁻ cells. The revised manuscript now presents a cohesive model in which *Trpv2* expression spans multiple early trophoblast states within the developing labyrinth and becomes progressively enriched in SynTII and S-TGC lineages, without implying exclusionary or lineage-ambiguous cell populations.

Additional comments:

- It is highly unlikely that all the *Synb*⁻ *Trpv2*⁺ cells are SynTII precursors. Visually, the overlap of the ISH signal with *Slc16a1* is much higher than with *Synb*, perhaps locating to different aspects of the same cells. Although the double-ISH stainings are extremely nice, the visualization of mRNA leaves room for ambiguity. Attempts should be made to better resolve cell type-specific expression in a clearer way, perhaps by ISH combined with IF. This might help establish the cell lineage relationships. Ideally, lineage tracing of *Trpv2*-expressing cells would be required to resolve the question of what cells /progenitors precisely express this gene.

We wish to clarify the reviewer’s comment regarding apparent overlap with *Slc16a1*. In our analyses, *Trpv2* shows little systematic overlap with *Slc16a1* (SynTI marker), (as documented in Supplementary Figure 9). We suspect that the reviewer may have intended

Slc16a3 (MCT4), which marks SynTII cells and indeed shows substantial spatial overlap with *Trpv2* in differentiating SynTII domains. To minimize subjective interpretation of co-localization in dense placental tissue, the original manuscript already included masked RNAscope images in which *Trpv2* signal is visualized exclusively within regions positive for specific lineage markers (e.g. *Synb*, *Pecam1*). These masked images (Figure 2 and Supplementary Figure 9) demonstrate that *Trpv2* is not enriched within *Slc16a1*⁺ SynTI compartments.

Rather than further extending static marker-based co-localization approaches or lineage tracing, both of which are technically challenging in syncytial placental tissues, we opted for MERscope-based spatial transcriptomics as the most appropriate method to resolve this issue further. As detailed above, these analyses demonstrate that *Trpv2*⁺/*Synb*⁻ regions do not represent a single, ill-defined precursor population, but instead correspond to spatially distinct early trophoblast domains that align either with SynTII differentiation or with an S-TGC-biased trajectory. Accordingly, the revised manuscript no longer interprets *Trpv2*⁺/*Synb*⁻ cells as ambiguous precursors, and we believe this directly addresses the reviewer's concern.

- The suspicion around the SynTII assurance is corroborated by data in Suppl. Fig. 14b that show no response in *Trpv2* expression to CHIR, hence a non-SynTII-associated expression pattern.

We thank the reviewer for this insightful comment. We agree that the lack of an overt change in *Trpv2* expression following CHIR-induced differentiation, as shown in Supplementary Figure 14b (now Supplementary Figure 18b), argues against a simple interpretation of *Trpv2* as a SynTII-exclusive marker. As stated in previous work and in the manuscript, *Trpv2* is expressed in multiple trophoblast subtypes, including several trophoblast giant cell populations (e.g. *Prl3d1*⁺ primary and secondary parietal TGCs, SpA-TGCs), SynTII cells, and S-TGCs. Consistent with this broad expression profile, we did not observe a net change in *Trpv2* expression levels when comparing normal differentiation conditions to CHIR-induced SynTII-biased differentiation. We propose that this apparent lack of change reflects shifts in lineage composition rather than absence of lineage association. Under standard differentiation conditions, TGC populations, many of which express *Trpv2*, are abundant, whereas SynTII cells are comparatively sparse. In contrast, CHIR treatment strongly enriches for SynTII cells, which also express *Trpv2*, while concomitantly reducing the relative abundance of TGC lineages. As a result, increases in *Trpv2* expression within the SynTII compartment may be counterbalanced by reduced representation of *Trpv2*-expressing TGCs, leading to an overall unchanged *Trpv2* signal at the bulk level.

Importantly, this interpretation is consistent with our spatial analyses, which demonstrate that *Trpv2* expression spans multiple early labyrinth trophoblast states and aligns with both SynTII and S-TGC differentiation trajectories, rather than being confined to a single lineage. Accordingly, we have revised the text to clarify that *Trpv2* is not a SynTII-exclusive marker, but rather marks early trophoblast populations whose lineage composition evolves depending on differentiation context.

- How do other SynT precursor markers behave in the presence of CHIR? (Also, Suppl. Fig. 6c is in a different colour tone.)

As highlighted in two recent studies (Angelova *et al.*, *Nat Commun* 2025⁹; Ballasy *et al.*, *Dev Biol* 2025¹⁰), SynTII differentiation occurs rapidly upon induction and is preceded by expression of specific precursor markers, including *Abcb1*, and *Gjb2*. In response to this comment, and to accommodate specific questions of Reviewer 3, we performed bulk RNA-sequencing of WT mTSC at day 3 under normal and CHIR-induced differentiation conditions. Supplementary Figure 18d shows the heatmap of DEGs between WT stem cells and differentiated cells with or without CHIR, with specific annotation of SynTII precursors markers.

We further also performed bulk RNA-sequencing of WT and *Trpv2*-KO mTSCs. Given the limited sample size (n=3) and heterogeneity of *in vitro* differentiation, differential expression analysis did not yield strong single-gene DEGs. We therefore analyzed the behavior of established SynTII and SynTII-precursor gene sets defined in published single-cell studies (Marsh *et al.*). This analysis revealed that while WT and *Trpv2*-KO cells show similar expression of SynTII-associated genes under normal differentiation conditions, CHIR treatment induces a coordinated SynTII transcriptional program in WT cells that is attenuated in *Trpv2*-deficient cells.

This was adapted in the revised manuscript.

- Little is made of the RNA-seq data beyond confirmation of down-regulation of SynTII genes. Yet even at E14.5 before the onset of FGR, the placental transcriptome is highly enriched for GO terms related to immune cell activation. What precisely are these much more prominently de-regulated, upregulated genes (Fig. 3c, Suppl. Fig. 12a)? Do they contribute to the phenotype? How does that translate to particular cell type(s) in the placenta that are proportionately more abundant (or alternatively, what cell types highly over-express these genes), and what relationship is there between these genes and TRPV2 function as a Ca²⁺ channel?

We noted indeed that gene ontology analysis of bulk RNA-sequencing data revealed enrichment for immune- and interferon-associated pathways. To address potential concerns regarding immune involvement, we have now explicitly examined the genes driving these GO terms and demonstrate that they predominantly comprise interferon-responsive and stress-associated regulators rather than canonical immune cell markers (Supplementary Figure 13b-c). Mapping of these genes to a published placental single-nucleus atlas (Marsh *et al.*) indicates that they are also expressed within trophoblast populations, mostly SynTI-cells. Furthermore, MERscope spatial transcriptomic analysis of canonical T-cell, B-cell, and myeloid markers shows no enrichment or clustering in abnormal labyrinth regions (Supplementary Figure 13d). Together, these analyses clarify that immune-associated GO terms reflect trophoblast-intrinsic stress and signaling responses rather than immune cell infiltration. In addition to E14.5 bulk RNA-sequencing, we also analyzed transcriptomes of E16.5 placentas. While reduced expression of SynTII markers was confirmed at this stage, upregulated genes at E16.5 did not show significant enrichment for specific biological pathways such as immune-associated pathways. Given the role of TRPV2 as a calcium-permeable channel, loss of TRPV2 may perturb Ca²⁺-dependent trophoblast maturation and homeostasis, secondarily inducing stress-responsive transcriptional programs without directly engaging immune pathways.

These concerns were discussed in the manuscript.

- What happens to *Trpv2* mutant pups when wildtype and heterozygous littermates are removed? Does the lack of competition allow *Trpv2*^{-/-} pups to survive? Previous data have reported reduced viability but seemingly did obtain some surviving KO animals. Along these lines, are epiblast-specific KO mice fully viable, i.e. have they been assessed beyond term?

We thank the reviewer for raising this important question. We did not systematically remove wild-type or heterozygous littermates to test whether reduced competition improves *Trpv2*^{-/-} pup survival. Based on our observations, however, we consider this unlikely to rescue viability. Homozygous knockout pups that are present at or shortly after birth are typically severely growth-restricted and moribund, and are frequently found unresponsive or not suckling. In many cases, *Trpv2*^{-/-} pups are not recovered alive, consistent with perinatal lethality rather than postnatal competition as the primary cause of death. It is well documented that dams tend to neglect or remove non-viable offspring, which is also consistent with our observations. Importantly, the primary focus of this study is on early placental development and the role of TRPV2-mediated calcium signaling in trophoblast differentiation, rather than on postnatal survival dynamics. The severe perinatal outcome observed in *Trpv2*-deficient animals is therefore interpreted as a downstream consequence of impaired placental development, rather than as a phenotype arising from competitive disadvantage after birth.

Our findings are in line with the original report by Park *et al.* (2011), who showed that *Trpv2* knockout lethality is strongly exacerbated on a C57BL/6 background. After six generations of backcrossing onto C57BL/6, only ~2.5% of pups surviving to weaning were homozygous knockouts, necessitating backcrossing to a mixed or 129S background to obtain viable adults. In our colony, maintained on a C57BL/6 background, we identified only 12 surviving *Trpv2*^{-/-} animals over a period of approximately five years, indicating that survival is a rare event rather than a competitive phenomenon.

Regarding epiblast-specific deletion, we have followed two independent litters of *Trpv2* epiblast-specific knockout mice beyond birth until 9 weeks of age. These animals showed no differences in postnatal viability or body weight compared with control littermates. These data have now been added as Supplementary Figure 4b and demonstrate that *Trpv2* loss in embryonic tissues does not impair postnatal survival, in contrast to global deletion.

- The lectin/cytokeratin stainings are unclear, the blue is faint and at times 'washed out', in particular in Fig. 4. Please replace with better examples or other staining types. Also please label what is stained in which colour.

We apologise for the unclear picture and have replaced Figure 4, which is now Figure 5, with a better example of E16.5, including labels. The original images, with subsequent MCT1/MCT4 staining was moved to supplementary figure 19.

- What is in the interspaces of SynTI-only areas? Is maternal blood present? Presumably no fetal blood? What other cells occupy this space?

We thank the reviewer for this highly interesting question regarding the cellular composition of regions in which SynTI cells are present in the apparent absence of SynTII and fetal capillaries. Based on conventional histological and immunohistochemical analyses (Supplementary Figure 11), these interspaces most closely resemble maternal

blood spaces rather than intact exchange regions. Specifically, the areas stain positive for alkaline phosphatase, a marker of S-TGCs that line the maternal blood space. Within these regions, we frequently observe enucleated red blood cells, consistent with maternal blood. In some instances, lectin-positive structures are present, and in these areas we occasionally detect nucleated red blood cells, likely representing residual fetal blood elements. Importantly, we were able to positively identify SynTI cells by *Slc16a1* expression and S-TGCs by *Ctsq* expression within or bordering these regions, whereas fetal endothelial cells were largely absent.

To further characterize the cellular composition of these aberrant areas without relying solely on individual markers, we performed MERscope-based spatial transcriptomic analysis. This analysis confirmed the absence of coordinated fetal endothelial gene expression within these regions, while revealing enrichment for trophoblast-associated programs, including SynTI-associated expression and S-TGC-related signatures. These data support the conclusion that SynTI-only regions lacking SynTII and fetal capillaries correspond to areas where trophoblast architecture is preserved but normal vascular invasion and organization have failed, resulting in expansion of maternal blood space-like regions rather than functional exchange units.

- What time point was designated “term” in the mouse? E19.5? Were the females in labour?

By “term”, we refer to the time of birth (postnatal day 0, P0). To accurately record birth weights, cages were inspected twice daily, and pups were weighed immediately after delivery. In our colony, parturition typically occurred between E19.5 and E20.5. Consequently, “term” corresponds to postnatal day 0 rather than a defined embryonic stage.

- Given the relatively little expression of TRPV2 in ST of human placentas but prominent staining in villous mesenchymal cells, the down-regulation of TRPV2 in FGR placentas may originate from non-trophoblast cell types. Have the authors assessed histological sections of these placentas for TRPV2 expression to determine in which cell types it is depleted?

We fully agree with the reviewer that the observed down-regulation of *TRPV2* mRNA in placentas from pregnancies affected by fetal growth restriction does not necessarily reflect altered expression within the syncytiotrophoblast itself, given the prominent *TRPV2* signal detected in villous mesenchymal cells in human placental sections. Unfortunately, the placental samples used for this analysis were collected as part of the EVERREST clinical trial, for which only RNA samples were available, and no material suitable for histological or spatial analyses was collected.

As a result, we are unable to determine from these samples which specific placental cell types contribute to the reduced *TRPV2* expression observed in FGR placentas. We therefore acknowledge that the decreased *TRPV2* signal may originate from non-trophoblast compartments, such as the villous core, rather than from the syncytiotrophoblast layer itself. In the revised manuscript, we have accordingly toned down the interpretation of the human FGR data and revised the text to avoid attributing the observed changes to a specific placental cell type. We now present these findings more

cautiously as a potential association between reduced placental TRPV2 expression and severe early-onset FGR, without inferring cell type-specific or causal mechanisms.

This was changed in the text as followed:

“While TRPV2 expression was reduced in a subset of FGR cases, particularly those delivered preterm, no difference was observed between term FGR samples and gestational age-matched controls (Supplementary Fig. 21e). Interpretation of these findings is limited by differences in gestational age at delivery and the small number of early-onset cases available for analysis. Moreover, the heterogeneity in clinical characteristics across samples precludes definitive conclusions regarding a direct association between TRPV2 expression and FGR.”

Minor comments:

Figures:

- Figure 2 is very low resolution in the PDF.

We hope that this resolved in the new manuscript.

- Fig. 2h: there is considerable variability in the labyrinth phenotype, sometimes almost following a bimodal pattern. Is there a genetic explanation for this (e.g., *Trpv2* imprinting or allelic bias in the heterozygous uterus)?

We thank the reviewer for this thoughtful observation. To the best of our knowledge, *Trpv2* is not an imprinted gene, and we have no evidence supporting allelic bias or parent-of-origin effects. Consistent with this, we do not observe a bimodal distribution of fetal weights in heterozygous litters, which would be expected if imprinting or allelic dosage effects were a major contributor to the variability. Moreover, data obtained on a different genetic background (129S) indicate that litters derived from *Trpv2*^{+/+} × *Trpv2*^{-/-} crosses exhibit comparable fetal weights regardless of whether the knockout allele is maternally or paternally inherited (see *graphs*), further arguing against uterine allelic bias as an explanation.

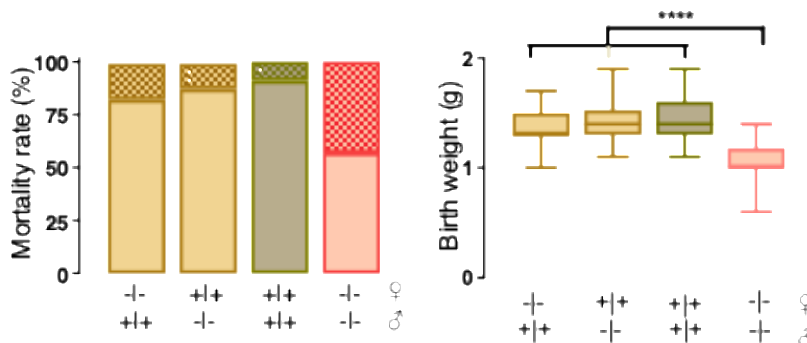


Figure 1: mortality rate and birth weight of heterozygous animals. Unlike homozygous crosses (+/+ X -/-) were monitored for perinatal lethality and birthweight in order to identify a possible dose-dependent and/or parental effects of TRPV2. Both crosses gave a similar average litter size of five pups. A mortality rate of 12 and 17% (26 from 202 and 34 from 195 pups, respectively) was observed in case the female was *Trpv2*^{-/-} or *Trpv2*^{+/+}, respectively (left). Nevertheless, there was no difference in birth weight of *Trpv2*^{+/+} mice compared *Trpv2*^{-/-} mice depending on the parental origin of the knockout allele (right), indicating that TRPV2 is unlikely to be parentally imprinted, **** $p < 0.0001$, One-way Anova with Turkey's multiple comparison test ($n = 47, 42, 46$ and 16 pups from $N = 8, 7, 7$ and 5 litters, respectively).

We therefore consider it unlikely that the apparent variability or bimodal pattern in the labyrinth phenotype reflects a genetic or imprinting mechanism. Instead, we believe that

the variability most likely reflects regional heterogeneity within the labyrinth itself and variability in the plane of sectioning. As shown by our MERscope analyses, morphologically normal and abnormal labyrinth regions can coexist within the same placenta. At present, we lack a three-dimensional reconstruction of the placenta that would allow us to determine how these regions are spatially distributed relative to one another. Consequently, individual histological sections may capture different proportions of normal versus abnormal regions, giving rise to the observed variability.

- Fig. 4d, overview image: Staining is of poor quality.

This has been replaced (see previous comments).

- UMAP plots: It would be helpful to label pertinent branches for easier identification, the colour scheme is very hard to make out.

Additional labels were added to the UMAP plot of figure 6.

- Suppl. Fig. 1c: The Sox2-Cre genotype is not relevant here.

We do find it relevant that from the F1 generation (female Sox2-cre x male *Trpv2^{F/F}*), we proceed further breedings with the Sox2.cre^{wt/wt}, and not the Sox2.cre^{wt/tg} because this would result in a different gene manipulation. Therefore, we opted to stress this again in Figure 1c.

- Suppl. Fig. 2c: Genes and rows are mis-aligned

We thank the reviewer for this observation and have now aligned these genes and rows properly.

- Supp. Fig. 7: typo in figure element annotation

The typo in Supplementary Figure 7 was corrected.

- Fig. 1 and Suppl. Fig. 3a: Nice diagrams, but the embryos and placentas are a little wonky, which in part might be a PDF conversion issue (some lines sticking out, the placental drawing could be improved). What is a (F)/+/- genotype? Also, what precisely is meant by the sentence in the legend, "Occasionally (8-12%), constitutive deletion was observed and represents full *Trpv2*^{-/-} mice, that were not assessed" – When was this observed (i.e. in which breeding strategy), how was it determined and how would constitutive deletion come about?

We thank the reviewer for these helpful comments regarding Figure 1 and Supplementary Figure 3a.

- First, we agree that the appearance of the schematic drawings may have been affected by PDF conversion. We have carefully re-checked and adjusted the original figure files to improve line rendering and overall clarity, particularly for the placental schematic.
- Second, the (F)/+/- genotype label has now been removed. This designation referred to rare mosaic recombination events that occasionally occurred during breeding. Animals with mixed or mosaic genotypes were never used for further breeding or phenotypic analyses and are no longer depicted in the revised figures to avoid confusion.
- Finally, constitutive deletion was observed specifically in the *Gcm1*-Cre breeding strategy, where sporadic, low-level expression of *Gcm1* occurs in male germ cells.

As demonstrated in Supplementary Figure 19c, this germline activity can result in recombination of the floxed *Trpv2* allele prior to fertilization, giving rise to embryos with ubiquitous deletion of *Trpv2* throughout the entire conceptus. These animals are referred to as *Gcm1.Trpv2^{-/-}* (*Gcm1-Cre^{wt/tg}*; *Trpv2^{-/-}*). This phenomenon allowed us, within the same litter, to directly compare embryos with complete *Trpv2* deletion (*Gcm1.Trpv2^{-/-}*) and those with SynTII-restricted deletion (*Trpv2^{SynTII-/-}* = *Gcm1-Cre^{wt/tg}*; *Trpv2^{F/-}*). Importantly, in contrast to global *Trpv2* knockout mice generated using maternal *Sox2-Cre*, the constitutive *Gcm1.Trpv2^{-/-}* embryos did not exhibit late embryonic lethality. This is consistent with the interpretation that late lethality is unlikely to occur in the SynTII-specific knockout and can be observed in the genotyping of the F1 generation (Supplementary Figure 19d). Moreover, birth weights reductions were similar for constitutive and conditional *Trpv2* knockout pups, further supporting our conclusion that deletion of *Trpv2* in SynTII cells alone is sufficient to cause fetal growth restriction.

Text:

- Please correct C57BL/6 mouse strain nomenclature in a few instances.

We have now adapted the nomenclature in the text.

- Line 92: Fetal growth ‘velocity’ is a strange term, presumably fetal growth rates or weight gain is meant here.

We thank the reviewer for this suggestion. Fetal growth velocity was changed for fetal growth rates.

- Line 113: “Reconstituting TRPV2 function only in the extraembryonic tissues” – this is misleading, as the gene was not reconstituted in trophoblast, instead it was specifically deleted from the epiblast-derived tissues

We apologies for this unclarity and have adapted the text as followed:

“Preserving TRPV2 function only in the extraembryonic tissues, and not in the embryo, restored the Mendelian inheritance”

- Lines 156, 159: What do the authors mean by ‘cellular dysplasia’ or ‘labyrinth dysplasia’?

We assumed the term dysplasia to point to regions with abnormal cell growth or development, typically affecting cell organization. Because this might not be received well, we changed dysplasia for malformation.

- Line 172: Fig. 3a should presumably be Fig. 3b

This typo was corrected in the manuscript.

Reviewer #3:

Key results

The authors performed tissue specific and total KO of TRPV2 in mouse and found that it is essential in the placenta but not in the embryo – resulting in FGR and embryonic lethality in the total KO and with no effect in embryo-specific KO.

CRISPR-cas9 mediated deletion of *Trpv2* in mTSCs resulted in a reduction of SynT-II marker expression, which was confirmed using SynT-II specific KO in mouse that partially replicated the FGR phenotype.

The authors also investigated the expression of *Trpv2* in human placentas with FGR and preterm birth with FGR and found that *Trpv2* expression was significantly decreased in placentas with preterm birth and FGR but not term FGR (see further comments relating to this below).

Validity

The results are largely accurately and carefully described in the text, however the conclusions drawn are often exaggerated and fail to account for experimental limitations. This is particularly in relation to the human placenta data presented in the manuscript and to the claim that *Trpv2* is “indispensable” for SynT-II differentiation.

This manuscript requires significant revision and further experiments to strengthen the evidence presented.

We thank the reviewer for this important overall assessment and agree that some of our initial conclusions were overly strong, particularly with respect to the interpretation of the human placental data and the role of *Trpv2* in SynTII differentiation.

In response to this comment, we have substantially revised the manuscript to moderate our conclusions and better align them with the strength of the data. Specifically:

- **We have toned down the interpretation of the human placental data throughout the Results, Discussion, Abstract, and Title, and now present these findings as descriptive and exploratory, explicitly acknowledging limitations related to gestational age and cohort heterogeneity.**
- **We have revised the wording describing the role of *Trpv2* in SynTII differentiation, replacing stronger claims such as “indispensable” with more accurate phrasing reflecting a role in the efficient activation of SynTII-associated transcriptional programs.**
- **We have incorporated additional experimental data, including bulk RNA sequencing and gene set-based analyses, which provide a more robust and comprehensive assessment of SynTII differentiation at the level of coordinated transcriptional programs.**

Together, these changes ensure that our conclusions are appropriately cautious and fully supported by the data.

Significance

The key role of the placenta in normal development is frequently overlooked. Therefore, this study which investigates the role of a *Trpv2* in specific components of the mouse placenta is important. Defining the mechanisms which underly placental development and function, and how these may contribute to disease is an important area of work. This manuscript describes sophisticated genetic manipulation which allows the role of *Trpv2* to be studied in the different placental compartments. This adds important new information to our understanding of placental function.

Data and methodology

Mouse knock-outs:

The knock-out of *Trpv2* in the whole mouse or specific tissues is a well-designed and logical strategy. However, I have 2 significant reservations about aspects of this work.

We thank the reviewer for the overall positive appraisal of this work and appreciate the valid comments, as further discussed below.

Comments:

i) In the previous cited paper describing the generation and analysis of *Trpv2* knock-out mice there was a very notable background-dependent effect on the phenotype. Specifically, in a BL/6 background (as in the study reported here) there was a very high rate of embryonic death close to term. However, in a mixed (BL/6 x 129S) cross, survival was markedly improved (although not completely normal). Thus, the strong claims made here that TRPV2 is “indispensable” (line 72 and elsewhere) is not true in a mixed genetic background. Such background-dependent effects are not uncommon in gm mice but the conclusions need to reflect this.

We agree with the reviewer that the phenotype observed upon *Trpv2* deletion is strongly genetic-background dependent. As reported previously, global *Trpv2* knockout mice on a pure C57BL/6 background exhibit a high rate of late embryonic or perinatal lethality, whereas survival is substantially improved, though not fully normalized, on a mixed C57BL/6 × 129S background. Such background-dependent effects are well recognized for placental and developmental phenotypes.

Our study was performed entirely on a C57BL/6 background, where the requirement for *Trpv2* is most pronounced. We therefore agree that claims of *Trpv2* being universally “indispensable” across genetic backgrounds would be overly strong. To address this concern, we have carefully revised the manuscript to temper the language throughout. Specifically, we have replaced the term “*indispensable*” with “*critical*” or “*significantly contributes to*” at multiple locations, including the title, Results, and Discussion sections.

While the genetic modifiers that permit partial survival on alternative backgrounds are of great interest, dissecting these compensatory mechanisms falls outside the scope of the present study and is the subject of ongoing work in our laboratory. We believe the revised wording now accurately reflects both our experimental findings and the established background dependence of *Trpv2* function in placental development.

ii) The logical progression from complete knock-out and the rescue in epiblast specific ablation points very strongly to a placental (indeed a trophectoderm lineage) dependent phenotype. This is solid. The lack of an obvious effect when the loss is confined to the ectoplacental cone (Tpbpa+) lineage points strongly to the placental labyrinthine zone. However, when the loss is confined to Gcm1+ cells the effect is considerably weaker – there is no perinatal death and only an embryonic growth defect. This suggests that placental Gcm1-ve cells contribute to the phenotype in some way. This is not discussed, and it needs to be.

We thank the reviewer for this thoughtful and accurate interpretation of the genetic data. We agree that the observed phenotypic gradient, from global *Trpv2* knockout, to epiblast rescue, to lineage-restricted deletions, strongly supports a placental, and specifically trophoblast-dependent, requirement for *Trpv2*. We further agree that the relatively mild

phenotype observed upon deletion in *Gcm1*⁺ cells, compared to the global knockout, implies that additional placental trophoblast populations contribute to the phenotype.

As noted by the reviewer, restriction of *Trpv2* deletion to the ectoplacental cone (*Tpbpa*⁺ lineage) did not result in fetal growth restriction, whereas deletion in *Gcm1*⁺ cells resulted in growth impairment without perinatal lethality. In addressing this discrepancy, we would like to emphasize that, according to single cell/single nucleus datasets, *Trpv2* and *Gcm1* are not exclusively co-expressed. While *Gcm1* expression is largely confined to SynTII cells, not all SynTII cells express *Gcm1*, and *Trpv2* is additionally expressed in SynTII precursor populations and other labyrinth trophoblast lineages that lack *Gcm1* expression (as shown by our spatial analyses (Supplementary Figure 7a, box 2) and supported by single-cell sequencing datasets).

As a consequence, *Gcm1*-driven deletion might not eliminate *Trpv2* expression from all SynTII cells, nor from earlier SynTII precursor populations or other labyrinth trophoblast cells. We therefore propose that residual *Trpv2* expression in *Gcm1*-negative trophoblast populations may partially compensate for *Trpv2* loss in *Gcm1*⁺ cells, thereby attenuating the severity of the phenotype and preventing perinatal lethality.

We have now explicitly addressed this point in the discussion, where we state that the incomplete overlap between *Gcm1* and *Trpv2* expression, and the persistence of *Trpv2* in other labyrinth trophoblast populations, likely underlies the weaker phenotype observed in *Trpv2*^{SynTII^{-/-}} knockouts compared to global *Trpv2* deletion. This revision clarifies that the phenotype is not solely attributable to *Gcm1*⁺ cells, but instead reflects the broader contribution of multiple labyrinth trophoblast populations expressing *Trpv2*.

This was briefly discussed as followed:

“This difference in phenotypic severity likely reflects either strain-dependent differences in Trpv2 knockout severity or the broader expression of Trpv2 across trophoblast differentiation trajectories, including precursor populations and additional lineages such as S-TGCs, suggesting that TRPV2 contributes to placental development at multiple stages of trophoblast differentiation.”

The authors used CRISPR-cas9 mediated deletion of *Trpv2* in mTSCs which is well described. KO of *Trpv2* resulted in a reduction in SynT-II marker gene expression as measured by qPCR, however the data are rather noisy. In Fig 3h and S14c the differences observed are significant only for one marker – *Gcm1*. This is not sufficient to claim that SynT-II differentiation is impaired in the KO mTSCs.

We thank the reviewer for this important comment and agree that the initial qPCR data alone showed variability and that the effect was most robust for *Gcm1*. We have therefore revised this part of the manuscript and strengthened our analysis by including additional data. Therefore, we performed bulk RNA sequencing to comprehensively assess transcriptional changes associated with *Trpv2* loss during differentiation. Gene set-based analysis revealed that, while differences under standard differentiation conditions were relatively modest, activation of a coordinated SynTII transcriptional program upon CHIR99021 treatment was robust in control cells but significantly attenuated in *Trpv2*-deficient mTSCs (Figure 4g). This indicates that TRPV2 is required for efficient activation

of the SynTII differentiation program, particularly under conditions that promote this lineage.

Together, these additional analyses support a model in which *Trpv2* contributes to the efficient activation of SynTII differentiation programs rather than causing a complete block in differentiation, and we have revised the wording of our conclusions accordingly.

A SynT-I fusion defect was observed in the *Trpv2* KO in mouse placental labyrinth IHC in Fig 3b. However, *Trpv2* KO had no effect on the SynT-I markers measured in mTSCs or in mouse labyrinth. The authors should explain these results.

We thank the reviewer for this important comment. We agree that the apparent discrepancy between the observed SynTI fusion defect *in vivo* and the unchanged SynTI marker expression requires clarification. Based on existing literature, SynTI differentiation and fusion are separable processes, with fusion occurring only after interaction with SynTII cells, while marker expression is established independently. In this context, the presence of SynTI markers in both *Trpv2*-deficient placentas and mTSC cultures indicates that SynTI lineage specification is preserved.

We therefore interpret the observed phenotype as a secondary consequence of impaired SynTII formation. Specifically, the absence of SynTII cells in abnormal regions is likely to disrupt the cues required for SynTI fusion, resulting in the altered morphology observed *in vivo*, while not affecting SynTI marker expression.

We have clarified this point in the Discussion as follows:

“Notably, SynTI marker-positive cells are still present in these areas but fail to acquire the characteristic elongated and flattened syncytial morphology, consistent with previous reports that SynTI fusion depends on interaction with SynTII cells¹¹. Together, these observations suggest that impaired SynTII formation represents a primary defect, with secondary consequences for SynTI maturation and vascular organization.”

The observation that *Gcm1* is consistently decreased with *Trpv2* KO in different experimental modalities highlights that *Trpv2* may exert some upstream control over *Gcm1* expression. The authors should comment on this, especially considering the crucial role *Gcm1* plays initiating SynT-II differentiation.

We thank the reviewer for this insightful comment and agree that the consistent reduction of *Gcm1* across multiple experimental modalities is an important observation. *Gcm1* is a key regulator of SynTII differentiation, and its downregulation in *Trpv2*-deficient systems suggests that TRPV2 may act upstream of, or in parallel with, pathways controlling *Gcm1* activation.

Our *in vitro* data further indicate that this effect is particularly evident under CHIR99021-induced differentiation conditions, where a coordinated SynTII transcriptional program is induced in WT cells but is attenuated in *Trpv2*-deficient cells. This suggests that TRPV2 contributes to the efficient activation of differentiation programs that include *Gcm1*, rather than regulating *Gcm1* in isolation. At present, we cannot distinguish whether TRPV2 directly regulates *Gcm1* expression or whether the observed effect reflects a broader impact on lineage specification and signaling pathways, such as calcium-dependent or

Wnt-associated mechanisms. We have clarified this in the revised manuscript and now explicitly state that TRPV2 likely acts as a modulator of transcriptional program activation upstream of, or converging on, Gcm1-mediated SynTII differentiation.

“The transcription factor Gcm1, a central regulator of SynTII differentiation, was reproducibly downregulated across multiple experimental modalities, supporting the idea that TRPV2 acts upstream of, or in coordination with, the regulatory network controlling SynTII specification.”

Use of publicly available datasets:

The authors have efficiently utilised publicly available datasets to strengthen their claims and direct further experiments.

Human data:

The localisation of TRPV2 mRNA differ in the human and mouse placenta – in the mouse it is predominantly in the syncytium but in the human it is mainly in the villous core. One notable exception is in the boxed region (1) in Fig 5a in which the CGA negative cells which appear to syncytium are TRPV2 +ve. This is not a big problem but it warrants some comment in the discussion

We thank the reviewer for this insightful comment. We agree that the localization of TRPV2 mRNA differs between mouse and human placenta, with a predominantly syncytial signal observed in mouse, whereas expression in human samples appears more enriched within the villous core.

Regarding the *TRPV2*-positive, *CGA*-negative cells highlighted in Figure 5a, now Figure 6a, we agree that these cells morphologically resemble syncytiotrophoblast but lack *CGA* expression. One possible interpretation is that *TRPV2* expression may precede the acquisition of terminal syncytiotrophoblast markers such as *CGA*, suggesting that *TRPV2* could be expressed at earlier stages of syncytial differentiation. This interpretation is consistent with our *in vitro* data in mouse, in which *Trpv2* contributes to the efficient activation of Syncytiotrophoblast II-associated transcriptional programs.

We have revised the discussion to include this point and to explicitly acknowledge potential species-specific differences in *TRPV2* localization, as well as the possibility that *TRPV2* marks earlier differentiation states within the syncytiotrophoblast lineage.

“Our findings also highlight both similarities and differences between mouse and human placentation. In human placenta, TRPV2 expression is predominantly observed within the villous core, but is also detected in subsets of cells with syncytium-like morphology lacking CGA expression, suggesting that TRPV2 may be expressed prior to terminal syncytiotrophoblast differentiation. This is consistent with our findings in mouse and in vitro systems and supports the idea that TRPV2 marks early stages of syncytial lineage development rather than exclusively mature syncytiotrophoblast cells.”

L 235, the statement that the syncytium is “mainly responsible for nutrient transfer” needs to be removed – this layer is also a major site of hormone production - as the authors themselves show in Fig 5a (*CGA* mRNA is extremely abundant).

We agree with the reviewer this should be mentioned and have adapted the text accordingly.

L 252 TRPV2 a “defining feature”, this is a very strong statement and needs to be toned down.

We based this statement on the fact that it was found a marker gene (using FindMarker function in Seurat R package), but have toned down this statement as followed:

“TRPV2 was abundantly present in a subset of fusing villous trophoblast cells”

L 258 The claims about relationship between placental TRPV2 mRNA and human fetal growth restriction are weak. A major challenge when investigating the relationship between placental mRNA, (or protein etc) and pathological conditions is that such analyses are confounded by gestational age. I.e, many important pathologies (pre-eclampsia, fetal growth restriction etc) lead to early delivery so comparing such samples with samples collected at term is invalid. Samples need to be matched for gestational age but samples collected pre-term are by definition not normal. This is an insurmountable challenge in placental research. Although the authors clearly acknowledge this (line 306) their conclusions ignore this and need to be more cautious. Specifically, there is no difference in TRPV2 mRNA when comparing the term FGR cases and controls. Hence the data from the pre-term FGR cases should not be compared with the term AGA samples. This leaves the 4 term early-onset FGR cases and 4 is a very small sample size. The details of some maternal characteristics (age, parity, BMI and mode of delivery) and fetal sex, indicate considerable variation. Just because there are no “significant” differences in these indexes it does not mean the groups are uniform. This is particularly true when there are only 4 subjects in one group. In addition, no neonatal outcomes are described. Although the participants were recruited as part of the EVERREST trial, it is not clear which centre collected these samples. All this means that any conclusions drawn from these 4 placentas must be treated with considerable caution and they are of necessity, weak. However, the authors’ claims in the title and abstract are insufficiently cautious.

We thank the reviewer for this thoughtful and important comment, and fully agree that the interpretation of human placental data is inherently confounded by gestational age and clinical heterogeneity. We also agree that the absence of a difference in TRPV2 mRNA levels between term FGR cases and gestational age-matched controls limits the strength of any conclusions that can be drawn regarding an association with fetal growth restriction.

In light of this, we have substantially revised this part of the manuscript to more cautiously reflect the limitations of the dataset. We have moved these data to Supplementary Figure 21 and revised both the Results and Discussion sections to ensure that the interpretation remains descriptive rather than inferential.

We now explicitly state that no difference is observed in term FGR samples compared to controls, and that the small number of early-onset cases (n=4), combined with variability in maternal and fetal characteristics, precludes firm conclusions. We also acknowledge that alternative explanations, including differences in disease severity, underlying etiology, or compensatory mechanisms, cannot be resolved with the current dataset. Overall, these changes ensure that our conclusions are appropriately cautious and that the human data are presented as exploratory and supportive rather than definitive.

“Finally, although decreased TRPV2 expression was observed in early-onset fetal growth restriction, particularly in preterm cases, interpretation of these data is limited by gestational age

differences and clinical heterogeneity. We therefore consider these findings exploratory and refrain from drawing causal conclusions.”

For completeness, we note that the human samples were collected at University College London Hospital as part of the EVERREST study; however, detailed neonatal outcome data were not available for this cohort.

Suggested improvements

Quantifying cell fusion and synT-I and II markers (using IHC) in the differentiating WT and KO mTSCs would significantly strengthen the claim of impaired SynT-II differentiation. Additionally, it would allow investigation of whether synT-I fusion defects are also present in mTSCs as described in fc-void areas in the mouse labyrinth.

We thank the reviewer for this important suggestion. We agree that quantitative assessment of cell fusion would, in principle, provide additional insight into syncytiotrophoblast differentiation *in vitro*. We have explored this experimentally; however, robust quantification of fusion in differentiating mTSC cultures proved technically challenging due to the intrinsic variability and heterogeneity of this system. Under standard differentiation conditions, lineage specification is not tightly controlled, resulting in stochastic and inconsistent fusion outcomes that preclude reliable quantitative interpretation. We are currently optimizing approaches to more precisely control lineage specification *in vitro*, which will be required to assess fusion in a reproducible and interpretable manner.

Furthermore, it remains unclear whether the cellular interactions proposed *in vivo*, that is, the requirement for coordinated differentiation and physical interaction between SynTI and SynTII cells for proper fusion, are faithfully recapitulated *in vitro*. Directly addressing this question would require establishing systems in which SynTI and SynTII specification can be genetically or experimentally uncoupled, for example through targeted perturbation of key regulators such as *Syna* and *Synb*. Such approaches are beyond the scope of the present study but represent an interesting direction for future work.

Finally, the *in vivo* phenotype in *Trpv2*-deficient placentas is characterized by regional and partial defects rather than a complete absence of SynTII cells, further suggesting that subtle or heterogeneous defects may not be readily captured in an *in vitro* system with limited spatial organization.

Importantly, the central conclusions of the manuscript are supported by consistent *in vivo* phenotypes and concordant transcriptional and gene program–level analyses demonstrating impaired SynTII differentiation, which provide a more robust and comprehensive readout of lineage specification than fusion assays alone.

Minor comments:

The text is too small to read in Figure S2

We adapted the figure and increased the font size. Furthermore, given the relatively little added value of Supplementary Figure 2a, we removed this from the figure.

Stereology – the units used to report this are confusing. It should either be a “fraction” or if corrected for tissue volume, then a volume (mm³)

Figure 2 and Supplementary Figure 8: ‘density’ has been changed to ‘fraction’. The text was also adapted accordingly.

Line 214 Fig S15b – note Trpv2 is not “highly expressed” in the differentiating
This was corrected in the text.

L 217 Fig 4 – legend and labelling have errors, particularly 4b-d and why are the fetal weights shown as averages and placental weights as individual data points?

Figure 4, now Figure 5, has been extensively adapted, where all data is now shown as box plots including individual points and expected mendelian pattern.

L221 – It is impossible to see cell morphology at the magnification shown.

We agree that the resolution of the current images does not allow definitive assessment of nuclear number or formal classification of syncytial versus mononuclear cells. Our intention was not to rigorously define nuclear status, but rather to illustrate the overall morphological appearance of the abnormal tissue. In particular, the cells shown in the KO condition lack the characteristic elongated and flattened morphology typically associated with differentiated syncytiotrophoblast-like cells *in vivo*, and instead retain a more compact appearance. We have revised the text to clarify this point and removed any statements implying definitive assessment of mononuclear versus multinucleated status based on these images.

“Interestingly, SynT1 cells in areas lacking SynTII did not exhibit the typical elongated and flattened morphology associated with fully differentiated syncytiotrophoblast cells, but instead appeared more compact”

And

“SynT1 marker–positive cells were present but did not exhibit the characteristic elongated and flattened morphology associated with fully differentiated syncytiotrophoblast cells”

L 234 the images in Fig 5a are too small and it is not possible to see the stained cells in the villus core. Similarly, Figs. S16a and b are much too small.

We thank the reviewer for this comment and agree that the visibility of stained cells within the villus core was limited at the original magnification. To address this, we have revised Figure 5a (now Figure 6a) to include higher-magnification views of representative regions of the villus core. These enlarged panels more clearly display the localization of the stained cells and allow better assessment of the described features. The updated figures improve clarity while preserving the overall tissue context. We further decided to remove Supplementary Figure 16a and b as they did not add any added scientific value to the story, rather than diluting the message.

L 365 “stillborn” – are the authors sure these animals were not born live and died shortly after birth?
– I suspect not so please reword.

As the reviewer kindly noted, we are indeed not 100% sure that the animals are not born alive. We changed wording to ‘suffered from perinatal lethality’.

Line 419-420 – “antibody” was not detected with the streptavidin-HRP – it was the biotinylated lectin.

This was adapted in text.

L774 Fig 3g has some rendering issues, a colour key is not where it should be and extra lines are present.

These issues were fixed in the revised manuscript.

L789 Fig 4a – adding a column with the expected mendelian ratios would be very helpful for reading the figure.

We thank the reviewer for this suggestion. As suggested, we considered including expected Mendelian ratios to facilitate interpretation of the breeding data. In standard crosses, these would indeed be 25% for each genotype. It should be noted, however, in this specific model, the presence of *Gcm1*-cre introduces variability due to sporadic and unpredictable recombination in germline cells, which affects the expected distribution of genotypes. We therefore added a column with expected, based on theoretical expectations rather than empirical findings.

L811 – “independent differentiation protocols” and “suggestive” need rephrasing

This was replaced by independent differentiations and ‘as a proxy for’.

L817 – “Turkey” should be Tukey

This was adapted in text.

Clarity and context

The information provided about how public datasets were re-analysed is insufficient to replicate the analysis – adding this to the methods would be helpful

We apologize and have included more details about the analysis of public datasets.

The level of detail provided on the generation of the different KO mouse lines is very different between the different lines – this should be expanded.

We thank the reviewer for pointing out this discrepancy. More details were provided for the epiblast and junctional zone-specific knockouts.

References

Line 60-62: There is a reference missing for the role of Trpv2 channels in the cell.

A reference was added in the text.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

- 1 Dong, C. *et al.* A genome-wide CRISPR-Cas9 knockout screen identifies essential and growth-restricting genes in human trophoblast stem cells. *Nat Commun* **13**, 2548 (2022). <https://doi.org/10.1038/s41467-022-30207-9>
- 2 Park, U. *et al.* TRP vanilloid 2 knock-out mice are susceptible to perinatal lethality but display normal thermal and mechanical nociception. *J Neurosci* **31**, 11425-11436 (2011). <https://doi.org/10.1523/JNEUROSCI.1384-09.2011>
- 3 Zhu, D., Gong, X., Miao, L., Fang, J. & Zhang, J. Efficient Induction of Syncytiotrophoblast Layer II Cells from Trophoblast Stem Cells by Canonical Wnt Signaling Activation. *Stem Cell Reports* **9**, 2034-2049 (2017). <https://doi.org/10.1016/j.stemcr.2017.10.014>
- 4 Marsh, B. & Blelloch, R. Single nuclei RNA-seq of mouse placental labyrinth development. *Elife* **9** (2020). <https://doi.org/10.7554/eLife.60266>
- 5 De Clercq, K. *et al.* Mapping the expression of transient receptor potential channels across murine placental development. *Cell Mol Life Sci* **78**, 4993-5014 (2021). <https://doi.org/10.1007/s00018-021-03837-3>
- 6 Jin, J. *et al.* Deletion of Trpm7 disrupts embryonic development and thymopoiesis without altering Mg²⁺ homeostasis. *Science* **322**, 756-760 (2008). <https://doi.org/10.1126/science.1163493>
- 7 Schutz, A. *et al.* Trophoblast cell failure leads to peri-implantation lethality in Trpm7-deficient mouse embryos. *Cell Rep* **37**, 109851 (2021). <https://doi.org/10.1016/j.celrep.2021.109851>
- 8 Chubanov, V. *et al.* Epithelial magnesium transport by TRPM6 is essential for prenatal development and adult survival. *Elife* **5** (2016). <https://doi.org/10.7554/eLife.20914>
- 9 Angelova, D. M. *et al.* Single-cell RNA sequencing identifies CXADR as a fate determinant of the placental exchange surface. *Nat Commun* **16**, 142 (2025). <https://doi.org/10.1038/s41467-024-55597-w>
- 10 Ballasy, N., Apantaku, I., Dean, W. & Hemberger, M. Off to a good start: The importance of the placental exchange surface - Lessons from the mouse. *Dev Biol* **517**, 248-264 (2025). <https://doi.org/10.1016/j.ydbio.2024.10.009>
- 11 Nadeau, V. & Charron, J. Essential role of the ERK/MAPK pathway in blood-placental barrier formation. *Development* **141**, 2825-2837 (2014). <https://doi.org/10.1242/dev.107409>

Point-to-point rebuttal – part II

Reviewer #1

The authors have addressed all of my critical points in the revised manuscript.

Reviewer #2

This revised study by De Clercq and colleagues investigates the role of the Ca²⁺ channel TRPV2. Using a series of constitutive and conditional knockouts, they show that the fetal growth retardation and propensity to perinatal lethality are driven by a placental dysfunction phenotype that is introduced by Trpv2 deletion. Specifically, they show a defect in syncytiotrophoblast layer II formation in Trpv2 mutants in quite some exquisite detail. In line with a primary function of TRPV2 in this particular cell type, a SynT-II-specific ablation of Trpv2 recapitulates significant aspects of the phenotype. They also explore the function of TRPV2 in human trophoblast cells and conclude that it may play similar roles also in human placentation, even if this conclusion remains somewhat speculative.

The overall message of the manuscript has not changed from the first submission, but the level of detail in which the phenotype has been assessed is substantially bolstered. The RNAscope and MERfish-based spatial transcriptomics data are impressive. In particular, the subsetting into normal and abnormal placental regions in the spatial transcriptomics experiments allows for a highly detailed level of resolution. Collectively, this manuscript is now far better aligned with the target journal, and the authors should be commended for the efforts they made in this revision to address the various points raised by the reviewers. Some remaining, minor points should be fixed:

Introduction

Reviewer Comment

Line 51, normal functioning placentation – please re-word

Author response

This was adapted in the text.

Reviewer Comment

Line 52/53: Please refrain from referring to syncytiotrophoblast cells or even endothelial cells as being “invasive”. In the context of placental development, this is a very confusing terminology that has nothing to do with the trophoblast invasion associated with spiral artery remodelling, which is attributed to entirely different cell types both in mice and in humans. Instead, please describe this as “invaginating” fetal blood vessels into the chorionic trophoblast cell layer, in line with the morphological movements 9as opposed to single cells invading into a foreign tissue).

Equally in line 55/56, I suggest to delete the “characterized by their invasive properties” part of the sentence.

Author response

We thank the reviewer for highlighting this important terminology issue. We agree that the term “invasive” may cause confusion with extravillous trophoblast-mediated invasion and spiral artery remodelling. We have revised the text to describe the process as invagination of fetal blood vessels into the chorionic trophoblast layer and removed references to invasive properties of SynTII/primitive syncytiotrophoblast cells.

“While abnormal placental growth or function is a major cause of FGR^{4,5}, the molecular players required for normal placentation are poorly understood. In both human and rodent placentas, establishment of an efficient maternal-fetal exchange interface depends on the coordinated development of trophoblast and fetal vascular compartments. This process involves progressive invagination of fetal blood vessels into the chorionic trophoblast layer, generating the highly branched exchange structures that characterize the mature placenta.”

Reviewer Comment

Line 63, we don't know whether TRP channels "excel" in this process, rephrase to "function" or similar

Author response

We agree and have replaced "excel" with "function as sensors that transduce environmental signals into cellular responses."

Reviewer Comment

Line 66 and also Results, it remains unclear what proportion of *Trpv2* mutants survives. Please specify these numbers (1) from previous KO studies and (2) in the current study.

Author response

We thank the reviewer for this suggestion and agree that the survival data were not sufficiently clear. The manuscript has been revised to explicitly state both the findings of the original *Trpv2* knockout study and those obtained in the present study. Specifically, the Results section now notes that the original report described recovery of only 2.5% *Trpv2*^{-/-} animals on a C57BL/6 background, compared to the expected Mendelian frequency of 25% (ref. 14). In addition, we now clarify that although *Trpv2*^{-/-} fetuses were recovered at approximately Mendelian frequencies at E18.5, 25% (14/55) were already moribund or dead at this stage. By contrast, only 12 of 777 offspring (1.5%) recovered at birth were *Trpv2*^{-/-}, compared with the expected Mendelian frequency of 25%. These revisions clarify that fetal compromise is already evident before birth but deteriorates substantially during late gestation and the perinatal period.

Results

Reviewer Comment

The description of the genetics around the Sox2-cre transgene are very confusing, what is transmitted is the CRE protein via the oocyte, which results in the excision of floxed alleles even in the absence of the Sox2Cre transgene itself. Please simplify to this end. The phrase "full body heterozygous deficient animals" is an oxymoron, "heterozygous" will suffice.

Author response

We thank the reviewer for this helpful suggestion and agree that the description of the breeding strategy was unnecessarily complex. The manuscript has been revised to clarify that maternal Sox2-Cre-mediated recombination results from transient exposure to maternally derived Cre recombinase, leading to efficient excision of the floxed *Trpv2* allele in embryonic and extraembryonic lineages, irrespective of inheritance of the Sox2-Cre transgene. The breeding scheme description has been simplified accordingly. In addition, the term "full body heterozygous deficient animals" has been removed and replaced by "*Trpv2* heterozygous mice" throughout the manuscript. As suggested, the focus is now placed on the resulting constitutive deletion rather than inheritance of the Sox2-Cre transgene itself.

"A constitutive Trpv2 knockout mouse on a C57BL/6J background was generated using maternal Sox2-Cre-mediated excision of the floxed Trpv2 allele. Transient exposure to maternally derived Cre recombinase results in efficient deletion of the floxed allele in both embryonic and extraembryonic lineages, even in offspring that do not inherit the Sox2-Cre transgene (Supplementary Fig. 1a-b)."

Reviewer Comment

Also in Suppl Fig. 3, top panel, simplify this to a +/- intercross. The Sox2-Cre is irrelevant here. As mentioned in the previous review, for a reader with less knowledge about this particular Cre line, this will be confusing. In this type of cross, it does not matter which Cre deleter was used to accomplish the mutant allele.

Author response

We agree and have simplified the breeding scheme shown in Supplementary Fig. 3 to a *Trpv2*^{+/-} intercross.

Comment

Related to that, lines 116-117, just state that *Trpv2* was deleted from all epiblast derivatives using the *Sox2-cre* transgene, including the fetal endothelial cells of the placenta. Currently, the sentence implies that endothelial cells are *Sox2*-positive.

Author response

We thank the reviewer for pointing out this ambiguity. The text has been revised to clarify that *Sox2-Cre* mediates deletion in all epiblast-derived lineages, including the fetal endothelial cells of the placenta, while *Trpv2* expression remains intact in extraembryonic trophoblast lineages. The previous wording suggesting that fetal endothelial cells are *Sox2*-positive has been removed.

Reviewer Comment

If only 25% of *Trpv2*^{-/-} fetuses die (i.e. one quarter of the 25% by Mendelian ratios, so that would be 6.25% dying and 18.75% surviving), how does that result in 2% of born offspring mentioned a few lines previously? These numbers are hard to follow, please describe these numbers more clearly.

Author response

This remark was already addressed in a previous comment, and the text was adapted to clarify the numbers and reduce confusion. We agree that the relationship between fetal demise at E18.5 and recovery of knockout offspring at birth was unclear. The Results section has been revised to explicitly state that *Trpv2*^{-/-} fetuses were recovered at approximately Mendelian frequencies at E18.5, despite 25% already being moribund or dead, whereas only 1.5% of offspring recovered at birth were *Trpv2*^{-/-}. The revised text now clarifies that substantial additional mortality occurs between E18.5 and birth.

“Consistent with the original Trpv2 knockout report, which described recovery of only 2.5% Trpv2^{-/-} animals on a C57BL/6 background instead of the expected 25%¹⁴, a disturbed Mendelian inheritance pattern was observed at birth, together with reduced litter sizes (Table 1, Fig. 1a and Supplementary Fig. 1c-d). Accordingly, only 12 of 777 offspring (1.5%) recovered at birth were Trpv2^{-/-}, and the few surviving knockout pups were non-viable and exhibited markedly reduced birth weights (Fig. 1b). In contrast, Trpv2^{-/-} fetuses were recovered at approximately Mendelian frequencies at E18.5 (55/158 fetuses), indicating that loss of Trpv2 does not substantially affect survival during most of gestation. Nevertheless, fetal weight was significantly reduced already at E16.5 (-10%) compared with Trpv2^{+/+} and Trpv2^{+/-} littermates (Supplementary Fig. 1e), and growth restriction further worsened by E18.5 (-32%). Consequently, whereas Trpv2^{+/-} fetuses nearly doubled their body weight between E16.5 and E18.5 (+199%), Trpv2^{-/-} littermates gained substantially less weight (+152%). Concomitantly, placental growth was reduced at E16.5 and E18.5 (Fig. 1d and Supplementary Fig. 1f). Moreover, 37% of Trpv2^{-/-} fetuses at E16.5 and 91% at E18.5 weighted below the 10th percentile of Trpv2^{+/+} fetal weights (Supplementary Fig. 1g-h). Fetal demise was observed in 25% (14/55) of Trpv2^{-/-} fetuses at E18.5 (Fig. 1c and Supplementary Fig. 1e). Thus, although Trpv2^{-/-} fetuses remained present at near-Mendelian frequencies at E18.5, viability progressively declined towards term. Together, these findings demonstrate that complete loss of Trpv2 causes progressive late-gestation FGR associated with placental growth impairment, fetal demise and profound perinatal loss.”

Reviewer Comment

Line 100 – Concomitantly

Author response

The text was adapted accordingly.

Reviewer Comment

Supp 2b, there seems to be a gene name missing for the top row (i.e., it is presumably *Tpbpa*, third from the top, that is so highly enriched in placenta). The *Trpv2* enrichment in this heatmap is not very clear, it looks almost as abundant in bone marrow, for example. Fig. S2a is clearer.

Author response

We thank the reviewer for identifying this omission. The missing gene label has been added to Supplementary Fig. 2b. Supplementary Fig. 2b was generated using the TissueEnrich webtool following inclusion of *Trpv2* in a list of 115 labyrinth-associated genes obtained from the MGI database. The heatmap

displays only genes that fulfil the TissueEnrich enrichment criteria (i.e., classified as Tissue Enriched, Tissue Enhanced or Group Enriched). Thus, although *Trpv2* is not among the most strongly placenta-restricted genes in the dataset, its presence in the heatmap indicates that it met the enrichment criteria applied by the algorithm. We agree that the relative tissue expression pattern of *Trpv2* is more readily appreciated in Supplementary Fig. 2a and have clarified the description of Supplementary Fig. 2b in the figure legend.

Reviewer Comment

Fig. S8b, e, please label the trajectories on the UMAP, the colour-code is indiscernible

Author response

We thank the reviewer for this suggestion. To improve readability, the major developmental trajectories and annotated cell populations have now been labelled directly on the UMAPs in Supplementary Fig. 8b,e. These annotations facilitate identification of the principal developmental branches and terminal cell states without relying solely on the colour-coded legends.

Reviewer Comment

Fig. S10g, what happened to the mutant placenta photo? What are all the background pixels? Please provide a better photo or histological specimen.

Author response

We thank the reviewer for pointing this out. Upon revisiting the figure, we noticed that image artefacts were introduced during figure export/PDF conversion, affecting the appearance of the mutant placenta image in Supplementary Fig. 10g. The panel has been replaced with a corrected high-quality image, free of these artefacts, in the revised manuscript.

Reviewer Comment

Line 185, "given the absence of *Tpbpa* expression"

Author response

The text was adapted accordingly.

Discussion

Reviewer Comment

Even if Word counts may be limited, please allude to a potential mechanism of how a Ca^{2+} channel could affect determination and differentiation of one particular trophoblast cell type. Are PIP2 augmentation and actin depolymerization (or other processes associated with *Trpv2*) specifically important for SynT-II formation.?

Author response

We thank the reviewer for this insightful suggestion. We agree that the mechanisms linking TRPV2 activity to SynT-II differentiation warrant further discussion. The Discussion has been expanded to briefly address potential downstream processes associated with TRPV2 function, including calcium-dependent signalling, cytoskeletal remodelling and phosphoinositide-dependent membrane dynamics. However, we also note that the apparent lineage specificity remains difficult to explain. Although TRPV2 is highly enriched in SynT-II cells, expression is also observed in other trophoblast populations, including primary and sinusoidal trophoblast giant cells, which appear considerably less affected by *Trpv2* deletion. At present, there is no clear evidence that known TRPV2-associated processes, such as actin remodelling or phosphoinositide signalling, are uniquely required for SynT-II differentiation. Interestingly, a similarly selective trophoblast phenotype has recently been reported following deletion of other broadly expressed genes, suggesting that certain trophoblast lineages may be particularly sensitive to perturbation of common signalling pathways. As these mechanisms were not directly investigated in the current study, we present them as intriguing hypotheses for future investigation. This was added to the discussion as followed:

“The selective vulnerability of the SynTII lineage to Trpv2 deletion remains intriguing. Although TRPV2 expression is highly enriched in SynTII cells, expression is also detected in other trophoblast populations, including primary and sinusoidal trophoblast giant cells, which appear comparatively less affected by Trpv2 loss. Furthermore, processes commonly associated with TRPV2 activity, such as calcium-dependent signalling, cytoskeletal remodelling and membrane dynamics, are not known to be uniquely required for SynTII differentiation. Interestingly, selective defects affecting discrete trophoblast lineages have also been reported following perturbation of other broadly expressed regulators. Whether SynTII cells possess a particular dependence on TRPV2-mediated calcium signalling, or whether TRPV2 interfaces with lineage-specific regulatory networks centered on Gcm1, remains to be established.”

Materials and Methods

Reviewer Comment

Related to Fig. 4, why is the empty vector control called “scr”? The methods don’t state that a random 20nt sequence was used as control. Please re-label Fig. 4 accordingly (“vec”, “EV”, “Ctrl” or similar). Were the controls also single cell-derived clones?

Author response

We thank the reviewer for this comment. The designation “scr” refers to a scrambled non-targeting gRNA control rather than an empty vector control. The sequences of the scrambled guides were included in Supplementary Table 2 (Scrambled_1: GCTTTCACGGAGGTTTCGACG; Scrambled_2: ATGTTGCAGTTCGGCTCGAT), but this was not sufficiently clear in the Methods section. The Methods have now been revised to explicitly state that non-targeting scrambled gRNAs were used to generate the control clones. In addition, both the scrambled control and *Trpv2*-targeted lines were generated through the same CRISPR/Cas9 workflow, including isolation and expansion of single-cell-derived clones. We have clarified these points in the revised manuscript.

Reviewer #3

The comments that the reviewer considered addressed are not discussed further below. Only the remaining points are addressed.

Reviewer Comment

Line 43 in the abstract still states “critical” which is still too strong and see further comment below.

Author response

We thank the reviewer for pointing this out. The term "critical" has been removed from the Abstract and replaced by more cautious wording that better reflects the data and avoids implying an absolute requirement for TRPV2 in placental development.

Reviewer Comment

Discussion now covers incomplete Gcm1/Trpv2 overlap and residual Trpv2 in Gcm1-negative SynTII-lineage and other labyrinth populations (L527–533). This is an improvement but the added text makes no mention of the GCM1 heterogeneity which is described in the rebuttal. Furthermore, the single cell/nuc data is at a fixed point in time whereas the Gcm1 driven cre only needs to be transiently active to ablate the Trpv2. I suggest the authors add a comment about the possible lack of recombination which could be related to the level and/or duration of cre expression and the chromatin architecture around the Trpv2 locus.

Author response

We thank the reviewer for this insightful suggestion. The Discussion has been expanded to acknowledge that the milder phenotype observed in *Trpv2^{SynTII-/-}* placentas may also reflect incomplete recombination. Specifically, we now note that Cre-mediated recombination efficiency can be influenced by the level and duration of Cre expression as well as chromatin accessibility at the *Trpv2* locus, potentially resulting in heterogeneous deletion within the targeted lineage. This was added in the discussion as followed:

*“In addition, incomplete recombination may have attenuated the phenotype observed in *Trpv2^{SynTII-/-}* placentas, as Cre-mediated recombination efficiency can be influenced by both the level and duration of Cre expression and by accessibility of the *Trpv2* locus.”*

Reviewer Comment

Cautious reframing confirmed in Results/Discussion (L575–576, L440-446); states no difference in term FGR, n=4 limitation acknowledged, findings called exploratory. This is an improvement. However, line 81 states “Finally, human placental TRPV2 expression was reduced in cases of early-onset fetal growth restriction (FGR) compared to appropriately grown pregnancies, suggestive of a potential association.” This is again a strong statement and is not a fair reflection of the data – It is only when early onset FGR with pre-term delivery samples are compared to AGA with term delivery that a difference is noted. This could be solely due to a difference in GA. This is an insurmountable problem and I suggest the authors simply remove the text in lines 81-83.

Author response

Lines 81-83 were removed from the revised manuscript, and similar statements were removed from the abstract.

Reviewer Comment

Regarding the in vitro fusion assays - These are informative points and adding some of this to the manuscript would be helpful and aid a reader's understanding of the complexity. I agree that these experiments could be challenging are outside the scope of this manuscript

Author response

We thank the reviewer for this constructive suggestion. While the proposed experiments remain outside the scope of the current study, we agree that the considerations raised regarding the complexity of trophoblast differentiation and syncytiotrophoblast fusion provide important context. We have therefore incorporated a brief discussion of the limitations of current *in vitro* differentiation systems and the challenges associated

with modelling coordinated SynTI-SynTII interactions in culture. This was added to the discussion as followed:

“Interpretation of trophoblast differentiation studies performed in vitro also requires caution. Under standard differentiation conditions, mTSC cultures generate heterogeneous trophoblast populations, making robust assessment of lineage-specific processes such as syncytial fusion challenging. Moreover, the coordinated interactions between SynTI and SynTII cells that are required for normal labyrinth formation in vivo are unlikely to be fully recapitulated in current in vitro systems. Future studies using more refined lineage-directed differentiation approaches may help distinguish direct effects of TRPV2 on trophoblast fate specification from secondary effects resulting from disrupted trophoblast cell-cell interactions. This may be particularly important given the regional nature of the placental defects observed in vivo, suggesting that local cellular interactions contribute to the phenotypic outcome following Trpv2 loss.”

Reviewer Comment

However, and this is comment rather than a request for a specific change, Figure 6 has a very large number of panels and which are therefore crowded and small. This does not make it an easy figure to follow

Author response

We thank the reviewer for this observation. We agree that Figure 6 contains a substantial amount of information. However, we elected to retain the integrated figure layout because the various analyses are mechanistically linked and separating them into multiple figures would fragment the presentation of the human placental analyses.

Reviewer Comment

Now Fig 3e text is too small.

Author response

We thank the reviewer for pointing this out and have adapted the figure to increase readability of Fig 3e.

Reviewer Comment

Lines 52-3 – This is odd wording: “extensive branching of chorionic trophoblast cells and subsequent invasion of fetal endothelial cells.” The trophoblast cells do not “branch” and do not invade the endothelial cells. Please re-word

Line 53- 56. This is unclear, it is true that trophoblast are invasive but it the syncytiotrophoblast in only invasive during very early implantation. Once the villi form, the surface – ie the syncytiotrophoblast is not invasive– it is the extravillous trophoblast (in humans) that are highly invasive and interact with the maternal endothelial cells (not the “fetal endothelial cells” as stated in line 53). This needs rewording and a little additional detail.

Author response

We agree with the reviewer and have rephrased this section to more accurately describe labyrinth formation and fetal vessel invagination, while avoiding terminology that could be confused with extravillous trophoblast-mediated invasion. The paragraph was reworded as:

“While abnormal placental growth or function is a major cause of FGR^{4,5}, the molecular players required for normal placentation are poorly understood. In both human and rodent placentas, establishment of an efficient maternal-fetal exchange interface depends on the coordinated development of trophoblast and fetal vascular compartments. This process involves progressive invagination of fetal blood vessels into the chorionic trophoblast layer, generating the highly branched exchange structures that characterize the mature placenta.”

Reviewer Comment

Line 110 "Remarkable, however, was the significant Trpv2 expression in the mouse placenta compared to other organs in the embryo or adult". Reword as: However, the significant Trpv2 expression in the mouse placenta compared to other organs in the embryo or adult was remarkable.

Author response

The suggested wording was used and adapted in the revised manuscript.

Reviewer Comment

Supplementary fig 10 I -for the labelling of the male and female placentas "sex" and not "gender" should be used.

Author response

We thank the reviewer for pointing this out and have adapted supplementary figure 10 accordingly.

Rebuttal letter responses

“Placental TRPV2 supports trophoblast function and normal fetal development.”

Validity — overall assessment

Reviewer comment

The results are largely accurately and carefully described in the text, however the conclusions drawn are often exaggerated and fail to account for experimental limitations. This is particularly in relation to the human placenta data presented in the manuscript and to the claim that *Trpv2* is “indispensable” for SynTII differentiation.

This manuscript requires significant revision and further experiments to strengthen the evidence presented.

Author response

We thank the reviewer for this important overall assessment and agree that some of our initial conclusions were overly strong, particularly with respect to the interpretation of the human placental data and the role of *Trpv2* in SynTII differentiation.

In response to this comment, we have substantially revised the manuscript to moderate our conclusions and better align them with the strength of the data. Specifically:

- We have toned down the interpretation of the human placental data throughout the Results, Discussion, Abstract, and Title, and now present these findings as descriptive and exploratory, explicitly acknowledging limitations related to gestational age and cohort heterogeneity.
- We have revised the wording describing the role of *Trpv2* in SynTII differentiation, replacing stronger claims such as “indispensable” with more accurate phrasing reflecting a role in the efficient activation of SynTII-associated transcriptional programs.
- We have incorporated additional experimental data, including bulk RNA sequencing and gene set-based analyses, which provide a more robust and comprehensive assessment of SynTII differentiation at the level of coordinated transcriptional programs.

Together, these changes ensure that our conclusions are appropriately cautious and fully supported by the data.

Reviewer: Addressed. Confirmed throughout: human data reframed as exploratory, “indispensable” softened. However, line 43 in the abstract still states “critical” which is still too strong and see further comment below. Bulk RNA-seq has been added and this supports the conclusions drawn.

Data and methodology — mouse knock-outs

Reviewer comment

The knock-out of *Trpv2* in the whole mouse or specific tissues is a well-designed and logical strategy. However, I have 2 significant reservations about aspects of this work.

Author response

We thank the reviewer for the overall positive appraisal of this work and appreciate the valid comments, as further discussed below.

Comment (i) — strain-background dependence of the phenotype

Reviewer comment

In the previous cited paper describing the generation and analysis of *Trpv2* knock-out mice there was a very notable background-dependent effect on the phenotype. Specifically, in a BL/6 background (as in the study reported here) there was a very high rate of embryonic death close to term. However, in a mixed (BL/6 x 129S) cross, survival was markedly improved (although not completely normal). Thus, the strong claims made here that TRPV2 is “indispensable” (line 72 and elsewhere) is not true in a mixed genetic background. Such background-dependent effects are not uncommon in gm mice but the conclusions need to reflect this.

Author response

We agree with the reviewer that the phenotype observed upon *Trpv2* deletion is strongly genetic-background dependent. As reported previously, global *Trpv2* knockout mice on a pure C57BL/6 background exhibit a high rate of late embryonic or perinatal lethality, whereas survival is substantially improved, though not fully normalized, on a mixed C57BL/6 x 129S background. Such background-dependent effects are well recognized for placental and developmental phenotypes.

Our study was performed entirely on a C57BL/6 background, where the requirement for *Trpv2* is most pronounced. We therefore agree that claims of *Trpv2* being universally “indispensable” across genetic backgrounds would be overly strong. To address this concern, we have carefully revised the manuscript to temper the language throughout. Specifically, we have replaced the term “*indispensable*” with “*critical*” or “*significantly contributes to*” at multiple locations, including the title, Results, and Discussion sections.

While the genetic modifiers that permit partial survival on alternative backgrounds are of great interest, dissecting these compensatory mechanisms falls outside the scope of the present study and is the subject of ongoing work in our laboratory. We believe the revised wording now accurately reflects both our experimental findings and the established background dependence of *Trpv2* function in placental development.

Reviewer: Addressed. Indispensable wording change confirmed in Abstract (L48), Discussion L591, and Fig. 1 legend (L1207); strain-dependence now discussed (L530).

Comment (ii) — weaker phenotype with *Gcm1*+ deletion

Reviewer comment

The logical progression from complete knock-out and the rescue in epiblast specific ablation points very strongly to a placental (indeed a trophectoderm lineage) dependent phenotype. This is solid. The lack of an obvious effect when the loss is confined to the ectoplacental cone (*Tpbpa*+) lineage points strongly to the placental labyrinthine zone. However, when the loss is confined to *Gcm1*+ cells the effect is considerably weaker — there is no perinatal death and only an embryonic growth defect. This suggests that placental *Gcm1*-ve cells contribute to the phenotype in some way. This is not discussed, and it needs to be.

Author response

We thank the reviewer for this thoughtful and accurate interpretation of the genetic data. We agree that the observed phenotypic gradient, from global *Trpv2* knockout, to epiblast rescue, to lineage-restricted deletions, strongly supports a placental, and specifically trophoblast-dependent, requirement for *Trpv2*. We further agree that the relatively mild phenotype observed upon deletion in *Gcm1*⁺ cells, compared to the global knockout, implies that additional placental trophoblast populations contribute to the phenotype.

As noted by the reviewer, restriction of *Trpv2* deletion to the ectoplacental cone (*Tpbpa*⁺ lineage) did not result in fetal growth restriction, whereas deletion in *Gcm1*⁺ cells resulted in growth impairment without perinatal lethality. In addressing this discrepancy, we would like to emphasize that, according to single cell/single nucleus datasets, *Trpv2* and *Gcm1* are not exclusively co-expressed. While *Gcm1* expression is largely confined to SynTII cells, not all SynTII cells express *Gcm1*, and *Trpv2* is additionally expressed in SynTII precursor populations and other labyrinth trophoblast lineages that lack *Gcm1* expression (as shown by our spatial analyses (Supplementary Figure 7a, box 2) and supported by single-cell sequencing datasets).

As a consequence, *Gcm1*-driven deletion might not eliminate *Trpv2* expression from all SynTII cells, nor from earlier SynTII precursor populations or other labyrinth trophoblast cells. We therefore propose that residual *Trpv2* expression in *Gcm1*-negative trophoblast populations may partially compensate for *Trpv2* loss in *Gcm1*⁺ cells, thereby attenuating the severity of the phenotype and preventing perinatal lethality.

We have now explicitly addressed this point in the discussion, where we state that the incomplete overlap between *Gcm1* and *Trpv2* expression, and the persistence of *Trpv2* in other labyrinth trophoblast populations, likely underlies the weaker phenotype observed in *Trpv2*^ΔSynTII^{-/-} knockouts compared to global *Trpv2* deletion. This revision clarifies that the phenotype is not solely attributable to *Gcm1*⁺ cells, but instead reflects the broader contribution of multiple labyrinth trophoblast populations expressing *Trpv2*.

This was briefly discussed as followed:

“This difference in phenotypic severity likely reflects either strain-dependent differences in Trpv2 knockout severity or the broader expression of Trpv2 across trophoblast differentiation trajectories, including precursor populations and additional lineages such as S-TGCs, suggesting that TRPV2 contributes to placental development at multiple stages of trophoblast differentiation.”

Reviewer: Partially addressed. Discussion now covers incomplete *Gcm1*/*Trpv2* overlap and residual *Trpv2* in *Gcm1*-negative SynTII-lineage and other labyrinth populations (L527–533)

This is an improvement but the added text makes no mention of the GCM1 heterogeneity which is described in the rebuttal. Furthermore, the single cell/nuc data is at a fixed point in time whereas the *Gcm1* driven cre only needs to be transiently active to ablate the *Trpv2*. I suggest the authors add a comment about the possible lack of recombination which could be related to the level and/or duration of cre expression and the chromatin architecture around the *Trpv2* locus.

CRISPR/Cas9 mTSC KO — noisy qPCR data

Reviewer comment

The authors used CRISPR-cas9 mediated deletion of *Trpv2* in mTSCs which is well described. KO of *Trpv2* resulted in a reduction in SynT-II marker gene expression as measured by qPCR, however the data are rather noisy. In Fig 3h and S14c the differences observed are significant only for one marker — *Gcm1*. This is not sufficient to claim that SynT-II differentiation is impaired in the KO mTSCs.

Author response

We thank the reviewer for this important comment and agree that the initial qPCR data alone showed variability and that the effect was most robust for *Gcm1*. We have therefore revised this part of the manuscript and strengthened our analysis by including additional data. Therefore, we performed bulk RNA sequencing to comprehensively assess transcriptional changes associated with *Trpv2* loss during differentiation. Gene set-based analysis revealed that, while differences under standard differentiation conditions were relatively modest, activation of a coordinated SynTII transcriptional program upon CHIR99021 treatment was robust in control cells but significantly attenuated in *Trpv2*-deficient mTSCs

(Figure 4g). This indicates that TRPV2 is required for efficient activation of the SynTII differentiation program, particularly under conditions that promote this lineage.

Together, these additional analyses support a model in which *Trpv2* contributes to the efficient activation of SynTII differentiation programs rather than causing a complete block in differentiation, and we have revised the wording of our conclusions accordingly.

Reviewer: Addressed. Bulk RNA-seq and gene-set analysis added showing a more widespread effect on SynTII lineage; conclusions reworded to “efficient activation” rather than a complete block (Results L354–363; Discussion L541–548; Fig. 4g).

SynT-I fusion defect vs. unchanged SynT-I markers

Reviewer comment

A SynT-I fusion defect was observed in the *Trpv2* KO in mouse placental labyrinth IHC in Fig 3b. However, *Trpv2* KO had no effect on the SynT-I markers measured in mTSCs or in mouse labyrinth. The authors should explain these results.

Author response

We thank the reviewer for this important comment. We agree that the apparent discrepancy between the observed SynTI fusion defect *in vivo* and the unchanged SynTI marker expression requires clarification. Based on existing literature, SynTI differentiation and fusion are separable processes, with fusion occurring only after interaction with SynTII cells, while marker expression is established independently. In this context, the presence of SynTI markers in both *Trpv2*-deficient placentas and mTSC cultures indicates that SynTI lineage specification is preserved.

We therefore interpret the observed phenotype as a secondary consequence of impaired SynTII formation. Specifically, the absence of SynTII cells in abnormal regions is likely to disrupt the cues required for SynTI fusion, resulting in the altered morphology observed *in vivo*, while not affecting SynTI marker expression.

We have clarified this point in the Discussion as follows:

“Notably, SynTI marker-positive cells are still present in these areas but fail to acquire the characteristic elongated and flattened syncytial morphology, consistent with previous reports that SynTI fusion depends on interaction with SynTII cells¹¹. Together, these observations suggest that impaired SynTII formation represents a primary defect, with secondary consequences for SynTI maturation and vascular organization.”

Reviewer: Addressed. Discussion now explains SynTI fusion as secondary to impaired SynTII formation (L510–513).

Gcm1 consistently decreased — possible upstream control by TRPV2

Reviewer comment

The observation that *Gcm1* is consistently decreased with *Trpv2* KO in different experimental modalities highlights that *Trpv2* may exert some upstream control over *Gcm1* expression. The authors should comment on this, especially considering the crucial role *Gcm1* plays initiating SynT-II differentiation.

Author response

We thank the reviewer for this insightful comment and agree that the consistent reduction of *Gcm1* across multiple experimental modalities is an important observation. *Gcm1* is a key regulator of SynTII differentiation, and its downregulation in *Trpv2*-deficient systems suggests that TRPV2 may act upstream of, or in parallel with, pathways controlling *Gcm1* activation.

Our *in vitro* data further indicate that this effect is particularly evident under CHIR99021-induced differentiation conditions, where a coordinated SynTII transcriptional program is induced in WT cells but

is attenuated in *Trpv2*-deficient cells. This suggests that TRPV2 contributes to the efficient activation of differentiation programs that include *Gcm1*, rather than regulating *Gcm1* in isolation. At present, we cannot distinguish whether TRPV2 directly regulates *Gcm1* expression or whether the observed effect reflects a broader impact on lineage specification and signaling pathways, such as calcium-dependent or Wnt-associated mechanisms. We have clarified this in the revised manuscript and now explicitly state that TRPV2 likely acts as a modulator of transcriptional program activation upstream of, or converging on, *Gcm1*-mediated *SynTII* differentiation.

“The transcription factor Gcm1, a central regulator of SynTII differentiation, was reproducibly downregulated across multiple experimental modalities, supporting the idea that TRPV2 acts upstream of, or in coordination with, the regulatory network controlling SynTII specification.”

Reviewer: Addressed. Discussion states TRPV2 acts upstream of / in coordination with the *Gcm1* regulatory network (L538–540).

Human data

Species difference in TRPV2 localisation; CGA-negative cells

Reviewer comment

The localisation of TRPV2 mRNA differ in the human and mouse placenta — in the mouse it is predominantly in the syncytium but in the human it is mainly in the villous core. One notable exception is in the boxed region (1) in Fig 5a in which the CGA negative cells which appear to syncytium are TRPV2 +ve. This is not a big problem but it warrants some comment in the discussion.

Author response

We thank the reviewer for this insightful comment. We agree that the localization of TRPV2 mRNA differs between mouse and human placenta, with a predominantly syncytial signal observed in mouse, whereas expression in human samples appears more enriched within the villous core.

Regarding the *TRPV2*-positive, *CGA*-negative cells highlighted in Figure 5a, now Figure 6a, we agree that these cells morphologically resemble syncytiotrophoblast but lack *CGA* expression. One possible interpretation is that *TRPV2* expression may precede the acquisition of terminal syncytiotrophoblast markers such as *CGA*, suggesting that *TRPV2* could be expressed at earlier stages of syncytial differentiation. This interpretation is consistent with our *in vitro* data in mouse, in which *Trpv2* contributes to the efficient activation of Syncytiotrophoblast II-associated transcriptional programs.

We have revised the discussion to include this point and to explicitly acknowledge potential species-specific differences in *TRPV2* localization, as well as the possibility that *TRPV2* marks earlier differentiation states within the syncytiotrophoblast lineage.

“Our findings also highlight both similarities and differences between mouse and human placentation. In human placenta, TRPV2 expression is predominantly observed within the villous core, but is also detected in subsets of cells with syncytium-like morphology lacking CGA expression, suggesting that TRPV2 may be expressed prior to terminal syncytiotrophoblast differentiation. This is consistent with our findings in mouse and in vitro systems and supports the idea that TRPV2 marks early stages of syncytial lineage development rather than exclusively mature syncytiotrophoblast cells.”

Reviewer: Addressed. Discussion addresses species-specific localisation and TRPV2 preceding terminal ST markers (L564–566). Fig. 6a now includes the higher-magnification insets showing these CGA-negative cells.

L235 — “mainly responsible for nutrient transfer”

Reviewer comment

L 235, the statement that the syncytium is “mainly responsible for nutrient transfer” needs to be removed — this layer is also a major site of hormone production - as the authors themselves show in Fig 5a (CGA mRNA is extremely abundant).

Author response

We agree with the reviewer this should be mentioned and have adapted the text accordingly.

Reviewer: Addressed. Now reads “nutrient transfer and hormone production” (L401–402).

L252 — “defining feature” too strong

Reviewer comment

L 252 TRPV2 a “defining feature”, this is a very strong statement and needs to be toned down.

Author response

We based this statement on the fact that it was found a marker gene (using FindMarker function in Seurat R package), but have toned down this statement as followed:

“TRPV2 was abundantly present in a subset of fusing villous trophoblast cells”

Reviewer: Addressed. Changed to “abundantly present in a subset of fusing villous trophoblast” (L425).

L258 — weak claims about TRPV2 mRNA and human FGR

Reviewer comment

L 258 The claims about relationship between placental TRPV2 mRNA and human fetal growth restriction are weak. A major challenge when investigating the relationship between placental mRNA, (or protein etc) and pathological conditions is that such analyses are confounded by gestational age. I.e, many important pathologies (pre-eclampsia, fetal growth restriction etc) lead to early delivery so comparing such samples with samples collected at term is invalid. Samples need to be matched for gestational age but samples collected pre-term are by definition not normal. This is an insurmountable challenge in placental research. Although the authors clearly acknowledge this (line 306) their conclusions ignore this and need to be more cautious. Specifically, there is no difference in TRPV2 mRNA when comparing the term FGR cases and controls. Hence the data from the pre-term FGR cases should not be compared with the term AGA samples. This leaves the 4 term early-onset FGR cases and 4 is a very small sample size. The details of some maternal characteristics (age, parity, BMI and mode of delivery) and fetal sex, indicate considerable variation. Just because there are no “significant” differences in these indexes it does not mean the groups are uniform. This is particularly true when there are only 4 subjects in one group. In addition, no neonatal outcomes are described. Although the participants were recruited as part of the EVERREST trial, it is not clear which centre collected these samples. All this means that any conclusions drawn from these 4 placentas must be treated with considerable caution and they are of necessity, weak. However, the authors’ claims in the title and abstract are insufficiently cautious.

Author response

We thank the reviewer for this thoughtful and important comment, and fully agree that the interpretation of human placental data is inherently confounded by gestational age and clinical heterogeneity. We also agree that the absence of a difference in *TRPV2* mRNA levels between term FGR cases and gestational age-matched controls limits the strength of any conclusions that can be drawn regarding an association with fetal growth restriction.

In light of this, we have substantially revised this part of the manuscript to more cautiously reflect the limitations of the dataset. We have moved these data to Supplementary Figure 21 and revised both the Results and Discussion sections to ensure that the interpretation remains descriptive rather than inferential.

We now explicitly state that no difference is observed in term FGR samples compared to controls, and that the small number of early-onset cases (n=4), combined with variability in maternal and fetal characteristics, precludes firm conclusions. We also acknowledge that alternative explanations, including differences in disease severity, underlying etiology, or compensatory mechanisms, cannot be resolved with the current dataset. Overall, these changes ensure that our conclusions are appropriately cautious and that the human data are presented as exploratory and supportive rather than definitive.

“Finally, although decreased TRPV2 expression was observed in early-onset fetal growth restriction, particularly in preterm cases, interpretation of these data is limited by gestational age differences and clinical heterogeneity. We therefore consider these findings exploratory and refrain from drawing causal conclusions.”

For completeness, we note that the human samples were collected at University College London Hospital as part of the EVERREST study; however, detailed neonatal outcome data were not available for this cohort.

Reviewer: Addressed. Cautious reframing confirmed in Results/Discussion (L575–576, L440–446); states no difference in term FGR, n=4 limitation acknowledged, findings called exploratory. This is an improvement. However, line 81 states “Finally, human placental TRPV2 expression was reduced in cases of early-onset fetal growth restriction (FGR) compared to appropriately grown pregnancies, suggestive of a potential association.” This is again a strong statement and is not a fair reflection of the data – It is only when early onset FGR with pre-term delivery samples are compared to AGA with term delivery that a difference is noted. This could be solely due to a difference in GA. This is an insurmountable problem and I suggest the authors simply remove the text in lines 81–83.

Suggested improvements

Quantify cell fusion and SynT-I/II markers in WT vs KO mTSCs

Reviewer comment

Quantifying cell fusion and synT-I and II markers (using IHC) in the differentiating WT and KO mTSCs would significantly strengthen the claim of impaired SynT-II differentiation. Additionally, it would allow investigation of whether synT-I fusion defects are also present in mTSCs as described in fc-void areas in the mouse labyrinth.

Author response

We thank the reviewer for this important suggestion. We agree that quantitative assessment of cell fusion would, in principle, provide additional insight into syncytiotrophoblast differentiation *in vitro*. We have explored this experimentally; however, robust quantification of fusion in differentiating mTSC cultures proved technically challenging due to the intrinsic variability and heterogeneity of this system. Under standard differentiation conditions, lineage specification is not tightly controlled, resulting in stochastic and inconsistent fusion outcomes that preclude reliable quantitative interpretation. We are currently optimizing approaches to more precisely control lineage specification *in vitro*, which will be required to assess fusion in a reproducible and interpretable manner.

Furthermore, it remains unclear whether the cellular interactions proposed *in vivo*, that is, the requirement for coordinated differentiation and physical interaction between SynTI and SynTII cells for proper fusion, are faithfully recapitulated *in vitro*. Directly addressing this question would require establishing systems in which SynTI and SynTII specification can be genetically or experimentally uncoupled, for example through targeted perturbation of key regulators such as *Syna* and *Synb*. Such approaches are beyond the scope of the present study but represent an interesting direction for future work.

Finally, the *in vivo* phenotype in *Trpv2*-deficient placentas is characterized by regional and partial defects rather than a complete absence of SynTII cells, further suggesting that subtle or heterogeneous defects may not be readily captured in an *in vitro* system with limited spatial organization.

Importantly, the central conclusions of the manuscript are supported by consistent *in vivo* phenotypes and concordant transcriptional and gene program-level analyses demonstrating impaired SynTII differentiation, which provide a more robust and comprehensive readout of lineage specification than fusion assays alone.

Reviewer: These are informative points and adding some of this to the manuscript would be helpful and aid a reader's understanding of the complexity. I agree that these experiments could be challenging are outside the scope of this manuscript

Minor comments

Figure S2 — text too small

Reviewer comment

The text is too small to read in Figure S2.

Author response

We adapted the figure and increased the font size. Furthermore, given the relatively little added value of Supplementary Figure 2a, we removed this from the figure.

Reviewer: Addressed.

Stereology units

Reviewer comment

Stereology — the units used to report this are confusing. It should either be a “fraction” or if corrected for tissue volume, then a volume (mm³).

Author response

Figure 2 and Supplementary Figure 8: ‘density’ has been changed to ‘fraction’. The text was also adapted accordingly.

Reviewer: Addressed.

L214 / Fig S15b — “highly expressed”

Reviewer comment

Line 214 Fig S15b — note *Trpv2* is not “highly expressed” in the differentiating.

Author response

This was corrected in the text.

Reviewer: Addressed.

L217 / Fig 4 — legend errors; averages vs individual points

Reviewer comment

L 217 Fig 4 — legend and labelling have errors, particularly 4b-d and why are the fetal weights shown as averages and placental weights as individual data points?

Author response

Figure 4, now Figure 5, has been extensively adapted, where all data is now shown as box plots including individual points and expected mendelian pattern.

Reviewer: **Addressed.**

L221 — cell morphology not visible at magnification

Reviewer comment

L221 — It is impossible to see cell morphology at the magnification shown.

Author response

We agree that the resolution of the current images does not allow definitive assessment of nuclear number or formal classification of syncytial versus mononuclear cells. Our intention was not to rigorously define nuclear status, but rather to illustrate the overall morphological appearance of the abnormal tissue. In particular, the cells shown in the KO condition lack the characteristic elongated and flattened morphology typically associated with differentiated syncytiotrophoblast-like cells *in vivo*, and instead retain a more compact appearance. We have revised the text to clarify this point and removed any statements implying definitive assessment of mononuclear versus multinucleated status based on these images.

“Interestingly, SynTI cells in areas lacking SynTII did not exhibit the typical elongated and flattened morphology associated with fully differentiated syncytiotrophoblast cells, but instead appeared more compact”

“SynTI marker-positive cells were present but did not exhibit the characteristic elongated and flattened morphology associated with fully differentiated syncytiotrophoblast cells”

Reviewer: **Addressed.** Text revised to describe compact vs elongated/flattened morphology and definitive nuclear claims removed (L386, L510).

L234 — Fig 5a and Figs S16a/b too small

Reviewer comment

L 234 the images in Fig 5a are too small and it is not possible to see the stained cells in the villus core. Similarly, Figs. S16a and b are much too small.

Author response

We thank the reviewer for this comment and agree that the visibility of stained cells within the villus core was limited at the original magnification. To address this, we have revised Figure 5a (now Figure 6a) to include higher-magnification views of representative regions of the villus core. These enlarged panels more clearly display the localization of the stained cells and allow better assessment of the described features. The updated figures improve clarity while preserving the overall tissue context. We further decided to remove Supplementary Figure 16a and b as they did not add any added scientific value to the story, rather than diluting the message.

Reviewer: **Addressed (Fig 6a).** However, and this is comment rather than a request for a specific change, Figure 6 has a very large number of panels and which are therefore crowded and small. This does not make it an easy figure to follow.

L365 — “stillborn”

Reviewer comment

L 365 “stillborn” — are the authors sure these animals were not born live and died shortly after birth? — I suspect not so please reword.

Author response

As the reviewer kindly noted, we are indeed not 100% sure that the animals are not born alive. We changed wording to ‘suffered from perinatal lethality’.

Reviewer: **Addressed.**

L774 — Fig 3g rendering issues

Reviewer comment

L774 Fig 3g has some rendering issues, a colour key is not where it should be and extra lines are present.

Author response

These issues were fixed in the revised manuscript.

Reviewer: Partially Addressed. Now Fig 3e text is too small.

L789 — Fig 4a expected Mendelian ratios

Reviewer comment

L789 Fig 4a — adding a column with the expected mendelian ratios would be very helpful for reading the figure.

Author response

We thank the reviewer for this suggestion. As suggested, we considered including expected Mendelian ratios to facilitate interpretation of the breeding data. In standard crosses, these would indeed be 25% for each genotype. It should be noted, however, in this specific model, the presence of *Gcm1*-cre introduces variability due to sporadic and unpredictable recombination in germline cells, which affects the expected distribution of genotypes. We therefore added a column with expected, based on theoretical expectations rather than empirical findings.

Reviewer: Addressed. Expected column present (legend shows exp: 25% / exp: 50%).

L811 — wording

Reviewer comment

L811 — “independent differentiation protocols” and “suggestive” need rephrasing.

Author response

This was replaced by independent differentiations and ‘as a proxy for’.

Reviewer: Addressed. Confirmed (L1311).

L817 — “Turkey” → Tukey

Reviewer comment

L817 — “Turkey” should be Tukey.

Author response

This was adapted in text.

Reviewer: Addressed.

Clarity and context

Public-dataset reanalysis under-described

Reviewer comment

The information provided about how public datasets were re-analysed is insufficient to replicate the analysis — adding this to the methods would be helpful.

Author response

We apologize and have included more details about the analysis of public datasets.

Reviewer: Addressed. Additional detail added in Methods (datasets section).

Uneven detail on KO line generation

Reviewer comment

The level of detail provided on the generation of the different KO mouse lines is very different between the different lines — this should be expanded.

Author response

We thank the reviewer for pointing out this discrepancy. More details were provided for the epiblast and junctional zone-specific knockouts.

Reviewer: Addressed. Methods expanded for epiblast and junctional-zone-specific KOs.

Line 60–62 — missing reference

Reviewer comment

References — Line 60-62: There is a reference missing for the role of Trpv2 channels in the cell.

Author response

A reference was added in the text.

Reviewer: Addressed. Reference added in the Introduction (~L70).

Additional minor points to be addressed:

Lines 52-3 – This is odd wording: “extensive branching of chorionic trophoblast cells and subsequent invasion of fetal endothelial cells.” The trophoblast cells do not “branch” and do not invade the endothelial cells. Please re-word

Line 53- 56. This is unclear, it is true that trophoblast are invasive but it the syncytiotrophoblast is only invasive during very early implantation. Once the villi form, the surface – ie the syncytiotrophoblast is not invasive– it is the extravillous trophoblast (in humans) that are highly invasive and interact with the maternal endothelial cells (not the “fetal endothelial cells” as stated in line 53). This needs rewording and a little additional detail.

Line 110 “Remarkable, however, was the significant Trpv2 expression in the mouse placenta compared to other organs in the embryo or adult”. Reword as: However, the significant Trpv2 expression in the mouse placenta compared to other organs in the embryo or adult was remarkable.

Supplementary fig 10 l -for the labelling of the male and female placentas “sex” and not “gender” should be used.