Assisted reproductive technologies induce temporally-specific placental defects and the preeclampsia risk marker sFLT1 in mouse.

Lisa A Vrooman, Eric A. Rhon-Calderon, Olivia Y. Chao, Duy K. Nguyen, Laren (Riesche) Narapareddy, Asha K. Dahiya, Mary E. Putt, Richard M. Schultz and Marisa S. Bartolomei
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Original submission

First decision letter

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MS TITLE: Assisted reproductive technologies induce temporally-specific placental defects and risk for preeclampsia in a mouse model.

AUTHORS: Lisa A Vrooman, Eric A Rhon-Calderon, Olivia Y Chao, Duy K Nguyen, Laren (Riesche) Narapareddy, Asha K Dahiya, Richard M Schultz, and Marisa S Bartolomei

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested (please also see Editor's note below), which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Editor's note

Please attend to the concerns:

Information of the number and size of litters in the experimental groups of this studies and the statistical analysis and discriminative power of the experimental design

The validity/limitation of evaluating the "grade effects" of the ART group/s against the naturally conceiving (no IVF-EC-ET) group

Coverage of previous works on the subject of the impact of ART on fetoplacental development

Reviewer 1

Advance summary and potential significance to field

The authors have carried out a longitudinal study (E12.5, E14.5, E18.5) of the impact of individual and combined assisted reproduction techniques (ART) on embryos and placentas with the comparator being pregnancies in naturally cycling mice. These are very challenging experiments. They report early (E12.5) effects of embryo transfer (ET) on fetal weight along with placental abnormalities, effects that normalized by E18.5. Embryos cultured in vitro were the most affected (as compared to the ET and superovulated groups) and showed both early (E12.5) effects on embryo weights and placental abnormalities as well as later (E18.5) effects on embryo weight, placental overgrowth (with region-specific effects) and increases in sFLT-1 (a protein implicated in preeclampsia in humans). The longitudinal placental histology and marker studies are particularly interesting.

Comments for the author

The study was carefully designed and conducted with most conclusions supported by the data presented. For the most part the authors are careful in extrapolating their mouse ART results to human, especially re-preeclampsia. Much more will need to be done to determine how relevant the findings presented here in the mouse ART model are to human preeclampsia. There are a few places in the text (e.g. Title, Abstract) where the links to preeclampsia may be overstated especially as the mouse model did not phenocopy all characteristics of human preeclampsia, in particular, blood pressure. Outlined below, I have a number of more specific Comments for the authors' consideration.

Specific Comments:
1-Title- I suggest modifying the title to better represent the findings and not to overstate the conclusions, e.g. "Assisted reproductive technologies induce temporally-specific placental defects along with the preeclampsia risk marker sFLT1 in a mouse model".

2-Abstract, lines 33-34- this sentence overstates the findings. Suggestion: Increases in sFLT-1 in this study suggest that IVF procedures have the potential to increase the risk for preeclampsia.

3-page 5, lines 85-88. This sentence needs to be reworded to reflect the results. In the ET group at E12.5, there was an effect on fetal weight and abnormalities were noted in placental vasculature. However, no experiments were done to show that the abnormalities in vasculature caused the decrease in fetal weight.

4-page 6, lines 122-124- In Fig. S3 (litter mean comparison), at E18.5, the IVF group (marked with a 'b') appears to have a lower fetal weight when compared to the naturally cycling group (marked with an 'a')

5-For most mothers there was a relatively modest increase in sFLT1 between the ET and IVF groups (Fig. 7E); the variability in the IVF group was particularly marked. The authors should consider discussing this in comparison to the types of increases seen in cases of preeclampsia in humans.

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6-To get a better appreciation of the differences between groups, I suggest adding a table to the supplemental material showing results for individual embryos/placentas in individual litters. In particular, it would be helpful to know individual litter sizes for each group and see numbers of embryos and resorptions as well as placental abnormalities for each embryo for each litter for each group. One explanation for the lack of increased blood pressure in mothers might be that only a fraction of the embryos in a given litter have placental abnormalities that would result in increased sFLT1.

7-Along the lines of comment 6 above, in previous work the authors have shown 'outlier' effects in their mouse ART studies. Can the authors comment on this aspect for the current studies. It is possible that litter numbers/group may be limiting here.

8-Figure S2- For ease of comparison, I suggest keeping the Y axis maximum values the same for the different groups.

9-Supplemental Material- S3 and S4-labelling for E12.5, E14.5 and E18.5 appears to be in the wrong place and should be moved to the right of A), B), C) for E12.5, the right of D), E), F) for E14.5 and to the right of G), H), I) for E18.5 for each of the figures.

Reviewer 2

Advance summary and potential significance to field

The authors conducted a longitudinal analysis to demonstrate the effect of specific ART procedures on eliciting placental abnormalities. They found that embryo culture is the major factor contributing to placental overgrowth, reduced fetal weight and reduced placental DNA methylation. Moreover, increased sFLT-1 implicated in causing preeclampsia. This study can deepen our understanding of causes of placental abnormalities resulting from in vitro embryo culture. Meanwhile, the study inspired us that using optimized embryo culture conditions at the ART process may help ensure a good pregnancy outcome.

Comments for the author

Specific concerns,

For Figure 6, it is better to additionally show the images of placenta sections using Prl8a8 in situ hybridization.

In line 330, the reference 14 cannot be founded.

The authors just test the expression level of sFLT-1 in circulation. It is hard to distinguish the correlation between sFLT-1 increase and preeclampsia-like symptoms without the in situ staining or hybridization in placenta sections.

It is necessary to further discuss the mechanism of decreased placental DNA methylation. Is the expression level of Dnmts lower in embryo culture conditions or the oxidation level of 5mC increased?

Reviewer 3

Advance summary and potential significance to field

This study describes experiments to evaluate the impact of various elements of the assisted reproduction process used in human IVF, on developmental changes in fetal and placental development, using a mouse model. The study adds to previous similar studies in two important ways; (1) using combination of different components of the IVF, embryo culture, and embryo transfer intervention, the study explores the relative degree of adverse impact of these different elements, and presents data showing that embryo culture, as opposed to the in vitro fertilization

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process, is a key cause of fetal growth and placental structural deficit, and (2) the study goes beyond previous studies to investigate structural correlates of placental function including expression of key development genes, sFlt1 production, area ratios of placental compartments, and specific epigenetic (methylation) changes in placental DNA. These are novel and informative analyses, that provide data consistent with an impact of embryo culture on methylation status in key placental genes, as well as elevated sFlt1 production.

Comments for the author

I have some major criticisms that should be addressed by undertaking further experiments, increased sizes of experimental groups, correct statistical analyses, and substantial additional information:

1. The groups sizes are very small with only n=3 recipient dams per group in many experiments. There are substantial dam effects on the key fetal weight and placental structure endpoints used in this study. There can be considerable between mother variation in average fetal and placental weight, implantation rate and litter size, that reflect maternal environmental exposures and events, and ultimately can skew data sets and impact the conclusions drawn. Many similar studies use 6-12 dams or more, to reduce the impact of between dam variation. I would recommend at least 6-8 dams per group to accommodate between-dam variance in these parameters.

2. The statistical analyses have not taken into account the impact on outcomes of (a) between-dam variance, and (b) litter size. The standard analysis for this type of experiment is to use dam as subject and litter size as covariate, in a mixed model analysis, showing data as estimated marginal mean. Because litter size is such an important determinant of fetal weight, and placental structure and function, this is critical.
Currently the analysis uses ANOVA with individual fetuses / placentae as subject. With correct analysis, the low n will be problematic and many of the current significant changes will likely be lost.

3. The authors do not explain how the gestational day (E) is calculated when embryo transfer has occurred – is E12.5, 14.5, and 18.5 defined relative to embryo age at transfer (eg. E3.5 + 9.0. 11.0 and 15.0 days after transfer), or relative to maternal tract age (eg. E2.5 +10.0, 12.0, and 16.0 days after transfer). If the later, how is the delayed embryo development and implantation that is known to occur after blastocyst transfer into earlier stages tracts, taken into account? Could it therefore account for the smaller fetal sizes seen in all 4 embryo transfer groups? Either way, how is it reasonable to compare these to the naturally conceived, in vivo developed group where there has been synchronous development between the embryo and tract, and no delay in development to accommodate embryo transfer?

4. It would be very helpful to include postnatal and adult outcomes for each group, along with length of gestation. This would indicate whether the adverse late gestation outcomes evolve into perinatal and postnatal losses, and also whether adverse metabolic programming effects are seen in offspring.

5. The immune response elicited by seminal fluid signalling factors is thought to influence maternal tract cytokines and endometrial receptivity, in such a way as to impact later fetal and placental development, in part by stimulating the female immune response to paternal alloantigens (Bromfield et al PNAS 2014; Watkins et al PNAS 2019). In the current study, dams in the naturally conceived group were exposed to the conceiving sire's (B6SJLF1) seminal fluid. In embryo transfer groups, recipient dams were prepared by mating with CD-1 vasectomised males, not B6SJLF1 males. Thus the tracts were exposed to different males that have different alloantigens and potentially different capacity to elicit a female tract cytokine and immune response.
Therefore, can the authors exclude the possibility that this variable contributes to the altered fetal and placental development seen in embryo transfer groups?

6. Embryo transfer was achieved by transcervical catheter (NSET device).
This intervention comprises a significant insult to the cervix that has potential to impact elements of maternal tract receptivity and capacity to support placental development. Given that the naturally conceived control.

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Group did not receive the NSET intervention, can the authors exclude the possibility that at least some of the adverse impact was due to this intervention?

7. Immunostaining data does not show a non-immune or isotype-matched negative control and no mention of this control is made in the materials and methods. How do the authors know that the staining is specific to the target protein? Rabbit immunoglobulin is notorious for non-specific binding.

8. The authors have included measurements of circulating maternal sFlt1 which in humans, is one marker of the clinical condition of preeclampsia. However, mice do not experience preeclampsia, and the presence of elevated circulating sFlt1 is not on its own sufficient to claim preeclampsia. Additionally elevated sFlt1 is a placental adaptation to preeclampsia, not a cause. The authors should be much more circumspect about claiming that IVF processes elevate the risk of preeclampsia by elevating sFlt1 (line 32-34).
9. In several places, the authors extrapolate directly to the human clinical setting and make strident and far-reaching claims about implications for human IVF outcomes (Eg. li 343-345; 392-394). These claims are not justified given the extensive differences between mouse and human reproduction, and IVF treatments and processes. While, if able to be sustained in larger data sets and with correct statistical analyses, the findings are very interesting and potentially informative for designing studies to investigate causes of poorer outcomes in IVF pregnancies, there would need to be substantial additional data from human studies to draw firm conclusions. The authors should be more circumspect about the relevance to the human clinical setting.

10. The question of how the IVF environment affects fetal and placental development has been already been investigated by many well-designed and informative studies. The authors have overlooked key papers from other authors that inform on impact of IVF, embryo transfer and embryo culture. These should be cited and the different outcomes discussed and reconciled with the current study.

First revision

Author response to reviewers' comments

Editorial notes: Please attend to the concerns:

Information of the number and size of litters in the experimental groups of this studies and the statistical analysis and discriminative power of the experimental design.
Detailed information on number and size of litters can now be found in Supplemental Table 2. Statistical analysis changes have been made following Reviewer 3's comments to use a mixed model that accounts for dam effects, which is described in lines 625-642. Further detail about sample size and discriminative power of the experimental design have been added to the Methods section (lines 484-491). Please note that incorporation of this new statistical model has led to differences in statistical significance. Changes to the Results section are highlighted in yellow.

The validity/limitation of evaluating the "grade effects" of the ART group/s against the naturally conceiving (no IVF-EC-ET) group
Discussion concerning the validity/limitation of comparing experimental groups to Natural, ET, or S control concepti has been added (lines 285-288). If the mixed model ANOVA was determined to be significant, pairwise statistical tests were conducted to determine differences among all groups not just comparison to the Natural controls. This approach allows insight into the contributions of ET and S procedures, as well as providing comparison among groups with similar litter size.

Coverage of previous works on the subject of the impact of ART on fetoplacental development
We have added a review of the impact of ART on fetoplacental development to the Introduction (lines 62-64), but have remained focused in our references that specifically investigated lVF as it pertains to the placental overgrowth and epigenetic reprogramming phenotypes in mouse using

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optimized IVF conditions (lines 65-68). Please see response to Reviewer 3 comment #10 for more detail.

Reviewer 1 Advance Summary and Potential Significance to Field:
The authors have carried out a longitudinal study (E12.5, E14.5, E18.5) of the impact of individual and combined assisted reproduction techniques (ART) on embryos and placentas with the comparator being pregnancies in naturally cycling mice. These are very challenging experiments. They report early (E12.5) effects of embryo transfer (ET) on fetal weight along with placental abnormalities, effects that normalized by E18.5. Embryos cultured in vitro were the most affected (as compared to the ET and superovulated groups) and showed both early (E12.5) effects on embryo weights and placental abnormalities as well as later (E18.5) effects on embryo weight, placental overgrowth (with region-specific effects) and increases in sFLT-1 (a protein implicated in preeclampsia in humans). The longitudinal placental histology and marker studies are particularly interesting.

Reviewer 1 Comments for the Author:
The study was carefully designed and conducted with most conclusions supported by the data presented. For the most part the authors are careful in extrapolating their mouse ART results to human, especially re-preeclampsia. Much more will need to be done to determine how relevant the findings presented here in the mouse ART model are to human preeclampsia. There are a few places in the text (e.g. Title, Abstract) where the links to preeclampsia may be overstated especially as the mouse model did not phenocopy all characteristics of human preeclampsia, in particular, blood pressure. Outlined below, I have a number of more specific comments for the authors' consideration.

Specific Comments:
1-Title- I suggest modifying the title to better represent the findings and not to overstate the conclusions, e.g. "Assisted reproductive technologies induce temporally-specific placental defects along with the preeclampsia risk marker sFLT1 in a mouse model".
We have edited the title as close to the Reviewer's suggestion, taking into account the 120 character limit for the journal. The current title is, "Assisted reproductive technologies induce temporally-specific placental defects and the preeclampsia risk marker sFLT1 in mouse." (lines 1-2).

2-Abstract, lines 33-34- this sentence overstates the findings. Suggestion: Increases in sFLT-1 in this study suggest that IVF procedures have the potential to increase the risk for preeclampsia.
In accordance with the Reviewer's suggestion, we have revised the sentence, which now reads, 'Increases in sFLT-1 observed in this study suggest that IVF procedures could increase the risk for preeclampsia.' (lines 44-46).

3-page 5, lines 85-88. This sentence needs to be reworded to reflect the results. In the ET group at E12.5, there was an effect on fetal weight and abnormalities were noted in placental vasculature. However, no experiments were done to show that the abnormalities in vasculature caused the decrease in fetal weight.
We have edited the sentence to change the implied causality.
Old text: 'Unexpectedly, we found embryo transfer alone led to reduced mid-gestation fetal weight due to impaired placental vasculature, although improvement in fetal weight and placental vasculature is observed by term."
Edited text: 'Unexpectedly, we found embryo transfer alone led to both impaired placental vasculature and reduced fetal weight at mid-gestation, although improvement in placental vasculature and fetal weight is observed by term." (lines 101-103).

4-page 6, lines 122-124- In Fig. S3 (litter mean comparison), at E18.5, the IVF group (marked with a 'b') appears to have a lower fetal weight when compared to the naturally cycling group (marked with an 'a')
We thank the reviewer for noting this error. The revised text now reads: 'These weight trends were similar when statistically compared by litter mean with the exception of S and EC fetal weight, which were no longer statistically different from Natural controls (Figure S4).' (lines 137-139).

5-For most mothers there was a relatively modest increase in sFLT1 between the ET and IVF

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groups (Fig. 7E); the variability in the IVF group was particularly marked. The authors should consider discussing this in comparison to the types of increases seen in cases of preeclampsia in humans.
We thank the reviewer for highlighting this important point. We have expanded this section of the Discussion.
Added text: "Our analysis of serum sFLT-1 levels indeed supports that IVF placentas produce significantly more sFLT-1 protein and that there is marked variability among dams in the IVF group when compared to ET. Increased sFLT-1 in IVF pregnancies has been noted in clinical studies (Lee et al., 2015; Sanchez et al., 2012). Importantly, our study suggests that some of the sFlt1 increase is independent of underlying infertility factors and can be specifically attributed to embryo culture. Although there is a well-established link between IVF and preeclampsia in humans, to date, very few clinical studies have assessed circulating sFlt1 levels in IVF pregnancies and no clinical studies have assessed sFlt1 expression or protein levels in the placenta. In the rodent model, there is more variability in sFLT1 levels among IVF individuals than the ET control group, suggesting a stochastic response to IVF procedures. Indeed, an increase in variability is observed in many of the morphological and epigenetic end points we assessed. Understanding the mechanisms underlying this stochasticity may explain why preeclampsia occurs with some IVF pregnancies but not others." (lines 357-371).

6-To get a better appreciation of the differences between groups, I suggest adding a table to the supplemental material showing results for individual embryos/placentas in individual litters. In particular, it would be helpful to know individual litter sizes for each group and see numbers of embryos and resorptions as well as placental abnormalities for each embryo for each litter for each group. One explanation for the lack of increased blood pressure in mothers might be that only a fraction of the embryos in a given litter have placental abnormalities that would result in increased sFLT1.
We agree with the reviewer that this information would be helpful to the reader. A Table of litter characteristics, noting the number of individuals, number of litters, the mean litter size, litter size range, percentage of live pups, and number of resorptions is now included as Supplemental Figure 2. We also agree that comparing individual concepti outcomes across the different phenotypes measured is helpful. Outcomes for individuals assessed for placental morphology and epigenetic assays is included as Supplemental Figure 9.

7-Along the lines of comment 6 above, in previous work the authors have shown 'outlier' effects in their mouse ART studies. Can the authors comment on this aspect for the current studies. It is possible that litter numbers/group may be limiting here.
We thank the reviewer for this suggestion and in response to the related comment #6, created a Table (Supplemental Figure 9) that would help readers keep track of individuals and allow us to comment on the question- Are the morphological and epigenetic abnormalities limited to certain individuals? Certain time-intensive assays with robust phenotypes did not necessitate assessment of all possible individuals, so the Table is limited to individuals that were assayed for ALL phenotypes. With respect to 'outliers', which remain vaguely defined, we only observed this in sFLT1 expression in the EC group at E18.5 (Figure 7C and Supplemental Figure 8).

8-Figure S2- For ease of comparison, I suggest keeping the Y axis maximum values the same for the different groups.
We agree with the reviewer and the graphs have been changed to have the same maximum value for the Y axis for the different experimental groups.

9-Supplemental Material- S3 and S4-labelling for E12.5, E14.5 and E18.5 appears to be in the wrong place and should be moved to the right of A), B), C) for E12.5, the right of D), E), F) for E14.5 and to the right of G), H), I) for E18.5 for each of the figures.
We appreciate the reviewer's finding of these errors. We have corrected the graphs by moving time point labels to the left of the correct corresponding graphs.

Reviewer 2 Advance Summary and Potential Significance to Field:
The authors conducted a longitudinal analysis to demonstrate the effect of specific ART procedures on eliciting placental abnormalities. They found that embryo culture is the major factor contributing to placental overgrowth, reduced fetal weight and reduced placental DNA

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methylation. Moreover, increased sFLT-1 implicated in causing preeclampsia. This study can deepen our understanding of causes of placental abnormalities resulting from in vitro embryo culture. Meanwhile, the study inspired us that using optimized embryo culture conditions at the ART process may help ensure a good pregnancy outcome.

Reviewer 2 Comments for the Author:
Specific concerns,

1- For figure6, it is better to additionally show the images of placenta sections using Prl8a8 in situ hybridization.
We thank the reviewer for this suggestion. We have edited Figure 6 to include representative images of *Prl8a8* in situ hybridization at E18.5, the time point when significant differences were observed (Figure 6G-K). The Figure legend lines have been edited to include their description (lines 1012-1014).

In line 330, the reference 14 cannot be founded.
We thank the reviewer for finding this typo. We have inserted the correct formatted reference (new in-text line 347, reference section lines 704-706).

The authors just test the expression level of sFLT-1 in circulation. It is hard to distinguish the correlation between sFLT-1 increase and preeclampsia-like symptoms without the in situ staining or hybridization in placenta sections.
We thank the reviewer for this suggestion but want to reiterate that although sFLT-1 was increased in the IVF group by comparison to ET controls, we did not observe significant differences in preeclampsia-like symptoms (i.e., maternal blood pressure). This failure is likely a limitation of the embryo transfer paradigm—ET and IVF litter size ranged from 1-9 pups; a 31-92% reduction compared to the Natural litter size expected for this breeding cross (average litter size is 13 pups from natural matings of CF1xB6SJLF1, with no embryo transfer). This detail has been added to the Discussion (lines 372-374). Although in situs or immunostaining can provide localization information, they do not provide a quantitative measure of overall expression or protein levels, making it difficult to make correlations.

It is necessary to further discuss the mechanism of decreased placental DNA methylation. Is the expression level of Dnmts lower in embryo culture conditions or the oxidation level of 5mC increased?
We have added what is known for this area of research in the Discussion (lines 399-408).
Added text: “How embryo culture results in loss of DNA methylation is poorly understood. Rabbit embryos cultured in two different media exhibited a reduction in *Tet1* and *Tet2* expression, genes that code for enzymes involved in active demethylation. No differences in *Dnmt1*, *Dnmt3a*, or *Dnmt3b*, genes coding for enzymes involved in maintenance and de novo DNA methylation, were detected (Salvaing et al., 2016). A study using human embryos observed abnormalities in DNMT3B levels and localization in poorer quality embryos compared to high quality embryos (Petrussa et al., 2014). Because all human embryo data come from patients undergoing some form of ART, these studies lack a non-ART control group, making it impossible to assess the full extent of ART effects.”

Reviewer 3 Advance Summary and Potential Significance to Field:
This study describes experiments to evaluate the impact of various elements of the assisted reproduction process used in human IVF, on developmental changes in fetal and placental development, using a mouse model. The study adds to previous similar studies in two important ways; (1) using combination of different components of the IVF, embryo culture, and embryo transfer intervention, the study explores the relative degree of adverse impact of these different elements, and presents data showing that embryo culture, as opposed to the in vitro fertilization process, is a key cause of fetal growth and placental structural deficit, and (2) the study goes beyond previous studies to investigate structural correlates of placental function including expression of key development genes, sFlt1production, area ratios of placental compartments, and specific epigenetic (methylation) changes in placental DNA. These are novel and informative analyses, that provide data consistent with an impact of embryo culture on methylation status in key placental genes, as well as elevated sFlt1 production.

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Reviewer 3 Comments for the Author:
I have some major criticisms that should be addressed by undertaking further experiments, increased sizes of experimental groups, correct statistical analyses, and substantial additional information:

1. The groups sizes are very small with only n=3 recipient dams per group in many experiments. There are substantial dam effects on the key fetal weight and placental structure endpoints used in this study. There can be considerable between mother variation in average fetal and placental weight, implantation rate and litter size, that reflect maternal environmental exposures and events, and ultimately can skew data sets and impact the conclusions drawn. Many similar studies use 6-12 dams or more, to reduce the impact of between dam variation. I would recommend at least 6-8 dams per group to accommodate between-dam variance in these parameters.
We agree with the reviewer that dam variance in natural conceptions can affect fetal and placental outcomes. However, our experimental paradigm (hormone stimulation, in vitro fertilization, embryo culture, and embryo transfer) affect the egg and/or preimplantation embryo, not the recipient dam. After embryo transfer, aspects of the experiment are highly controlled. Unlike natural conceptions, ET, S, EC, and IVF concepti do not show clustering by dam, strongly suggesting that the influence of dam is reduced after ET. This failure to cluster by dam is likely due to a few contributing factors, including, 1) concepti are generated from a mix of different egg (IVF group only) or embryo donors (ET, S, and EC groups), 2) litter sizes are small, which eliminates the crowding effect on fetal and placental weight that occurs when litters are large, and 3) robust effects reduce the impact of dam variance. The placental overgrowth phenotype we are studying has been independently observed by at least five different research groups (Bloise et al., 2012, Chen et al., 2015, Collier et al., 2009, Sui et al., 2014, and Tan et al., 2016), demonstrating it is reproducible and likely not due to dam variance. Nevertheless, we have still accounted for influence of dam in our mixed model statistical analysis to address this concern given that some of our analyses produced more subtle effects.

2. The statistical analyses have not taken into account the impact on outcomes of (a) between-dam variance, and (b) litter size. The standard analysis for this type of experiment is to use dam as subject and litter size as covariate, in a mixed model analysis, showing data as estimated marginal mean. Because litter size is such an important determinant of fetal weight, and placental structure and function, this is critical. Currently the analysis uses ANOVA with individual fetuses / placentae as subject. With correct analysis, the low n will be problematic and many of the current significant changes will likely be lost.
We have considered this point very carefully in our experimental design to ensure that dam variation was limited. All groups were generated concurrently and CF1 females were randomly assigned as Natural, ET, S, EC embryo donor, IVF egg donor, or pseudo-pregnant embryo transfer recipient. The analyses of multiple time points and multiple groups also helps eliminate any changes that are due to chance as trends can be tracked across experimental groups and across development. After discussing and bringing Dr. Mary Putt, a biostatistician, onboard, we approached analysis as the concepti as subject and dam effect as a covariate. The reviewer brings up an important point regarding the impact of litter size on outcome, particularly fetal and placental weight. We considered adjusting for litter size in the mixed effects model, but there is no overlap in the distribution of litter size between the natural group and the ART procedure groups. In the absence of overlap, a statistical adjustment for litter size would not be valid. This is an unfortunate limitation of the non-surgical embryo transfer mouse model, that although it is more clinically relevant, it leads to the inability to obtain comparable litter size to natural litters. Our understanding of the effect of litter size on fetal and placental development is that fetal and placental weight are negatively correlated with litter size and this phenomenon is only relevant to late gestation in mouse, when the majority of fetal growth occurs (McCarthy, 1967, Ishikawa et al. 2006). IVF concepti display the opposite trend for fetal weight-- smaller litter size had reduced fetal weight. Nevertheless, we have still accounted for influence of dam in our mixed model statistical analysis to address this concern.

3. The authors do not explain how the gestational day (E) is calculated when embryo transfer has occurred – is E12.5, 14.5, and 18.5 defined relative to embryo age at transfer (eg. E3.5 + 9.0. 11.0 and 15.0 days after transfer), or relative to maternal tract age (eg. E2.5 +10.0, 12.0, and 16.0 days after transfer). If the later, how is the delayed embryo development and

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implantation that is known to occur after blastocyst transfer into earlier stages tracts, taken into account? Could it therefore account for the smaller fetal sizes seen in all 4 embryo transfer groups? Either way, how is it reasonable to compare these to the naturally conceived, in vivo developed group where there has been synchronous development between the embryo and tract, and no delay in development to accommodate embryo transfer?
We thank the reviewer for this suggestion and have included these details in the Methods (lines 468-469). Gestational day was calculated relative to embryo age at transfer, (+9.0, 11.0, 15.0 days after transfer). Regarding the concern for delayed development/implantation due to embryo transfer, we do not discount that transfer into E2.5 maternal tract could factor into the observed differences in all four embryo transfer groups. However, this factor related to embryo transfer is also relevant to human IVF given that embryos are transferred into slightly different timed maternal environment and IVF pregnancies are often compared to a control group of spontaneous pregnancies. Importantly, our conclusion that EC and IVF groups are not able to normalize fetal weight and display placental overgrowth by term is unaffected by this concern.

4. It would be very helpful to include postnatal and adult outcomes for each group, along with length of gestation. This would indicate whether the adverse late gestation outcomes evolve into perinatal and postnatal losses, and also whether adverse metabolic programming effects are seen in offspring.
We agree with the reviewer that postnatal and adult outcomes are an outstanding question, but believe are beyond the scope of this manuscript. Studies of long-term outcomes of IVF procedures and how they may be linked to abnormal placentation/prenatal development are currently underway in our laboratory.

5. The immune response elicited by seminal fluid signalling factors is thought to influence maternal tract cytokines and endometrial receptivity, in such a way as to impact later fetal and placental development, in part by stimulating the female immune response to paternal alloantigens (Bromfield et al PNAS 2014; Watkins et al PNAS 2019). In the current study, dams in the naturally conceived group were exposed to the conceiving sire's (B6SJLF1) seminal fluid. In embryo transfer groups, recipient dams were prepared by mating with CD-1 vasectomised males, not B6SJLF1 males. Thus the tracts were exposed to different males that have different alloantigens and potentially different capacity to elicit a female tract cytokine and immune response. Therefore, can the authors exclude the possibility that this variable contributes to the altered fetal and placental development seen in embryo transfer groups?
We thank the reviewers for bringing this aspect to our attention. When discussing the embryo transfer effects, we have revised the text from 'embryo transfer alone' to 'factors related to the embryo transfer procedure' to be more inclusive (lines 141, 273-274, 289). We believe this isn't necessarily a limitation given that some women undergoing embryo transfer may not be exposed to seminal fluid from the conceiving male (e.g., sperm donor cycles).

6. Embryo transfer was achieved by transcervical catheter (NSET device). This intervention comprises a significant insult to the cervix that has potential to impact elements of maternal tract receptivity and capacity to support placental development. Given that the naturally conceived control. Group did not receive the NSET intervention, can the authors exclude the possibility that at least some of the adverse impact was due to this intervention?
Our experimental paradigm replicates as much as possible clinical procedures. To that end, the NSET device is used because transcervical catheters are used for embryo transfer in human IVF. The inclusion of the ET experimental group in all our experiments does allow comparison between Naturally conceived controls (no ET) and the other experimental groups. The data do indeed demonstrate that some abnormalities can be attributed to factors related to the embryo transfer procedure (significant decrease in fetal weight at E12.5 fetal placental ratio at E14.5 and E18.5, and *Ctsq* and *Slc16a3* expression) when compared with naturally conceived controls. Importantly, in most cases, embryo culture and IVF groups are still significantly different from ET concepti. Further, both the placental overgrowth and loss of imprinting E18.5 placental phenotypes have been independently observed by other research groups utilizing surgical embryo transfer protocols (Bloise et al., 2012, Chen et al., 2015, Collier et al., 2009, Sui et al., 2014, and Tan et al., 2016), which strongly suggests that transcervical embryo transfer is not important for late gestational placental overgrowth or loss of imprinting.

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7. Immunostaining data does not show a non-immune or isotype-matched negative control and no mention of this control is made in the materials and methods. How do the authors know that the staining is specific to the target protein? Rabbit immunoglobulin is notorious for non-specific binding.
We apologize for not describing negative controls in the Materials and Methods Immunostaining section in our initial submission and have included these details (lines 529-536).

8. The authors have included measurements of circulating maternal sFlt1 which in humans, is one marker of the clinical condition of preeclampsia. However, mice do not experience preeclampsia, and the presence of elevated circulating sFlt1 is not on its own sufficient to claim preeclampsia. Additionally elevated sFlt1 is a placental adaptation to preeclampsia, not a cause. The authors should be much more circumspect about claiming that IVF processes elevate the risk of preeclampsia by elevating sFlt1 (line 32-34).
This concern was also shared by Reviewer 1 and we have edited this sentence in line with the Reviewer 1's specific suggestion.
Edited text: '…and increased sFLT-1, an anti-angiogenic protein implicated in causing the maternal symptoms of preeclampsia in humans. Increases in sFLT-1 observed in this study suggest that IVF procedures could increase the risk for preeclampsia.' (lines 44-46).

9. In several places, the authors extrapolate directly to the human clinical setting and make strident and far-reaching claims about implications for human IVF outcomes (Eg. li 343-345; 392-394). These claims are not justified given the extensive differences between mouse and human reproduction, and IVF treatments and processes. While, if able to be sustained in larger data sets and with correct statistical analyses, the findings are very interesting and potentially informative for designing studies to investigate causes of poorer outcomes in IVF pregnancies, there would need to be substantial additional data from human studies to draw firm conclusions. The authors should be more circumspect about the relevance to the human clinical setting.
We have changed the language to highlight the experimental nature of mouse experiments and have further described the need for future clinical studies in the lines of concern:

Old text 343-345: 'Importantly our study demonstrates some of that increase is independent of underlying infertility factors and can be specifically attributed to the embryo culture procedure.',

Edited and added text (lines 361-366): Importantly, our study suggests that some of the sFlt1 increase is independent of underlying infertility factors and can be specifically attributed to embryo culture. Although there is a well-established link between IVF and preeclampsia in humans, to date, very few clinical studies have assessed circulating sFlt1 levels in IVF pregnancies and no clinical studies have assessed sFlt1 expression or protein levels in the placenta.

Old text 392-394: 'Our study also suggests that embryo culture procedures cause the most severe placental phenotypes associated with reduced fetal growth. Thus, measures to ameliorate adverse outcomes due to abnormal placentation in humans should focus on embryo culture optimization.'

Edited text 428-431: 'Our study also suggests that embryo culture causes the most severe placental phenotypes among the individual IVF procedures and is associated with reduced fetal growth. Thus, future research to improve embryo culture may ameliorate adverse outcomes due to abnormal placentation in humans.'
We made changes to Title and Abstract as suggested by Reviewer 1 (Comments 1 & 2).

10. The question of how the IVF environment affects fetal and placental development has been already been investigated by many well-designed and informative studies. The authors have overlooked key papers from other authors that inform on impact of IVF, embryo transfer and embryo culture. These should be cited and the different outcomes discussed and reconciled with the current study.
We understand the need for proper citation but also had concerns about readability of the manuscript as there is abundant literature on this topic. We have edited a portion of the introduction to provide more information and added a review citation of fetal and placental outcomes with ART in experimental animal models (Bloise et al. 2014). We chose to highlight

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references that specifically investigated IVF as it pertains to the placental overgrowth and epigenetic reprogramming phenotypes in mouse using optimized IVF conditions.

Edited text: "…numerous studies using fertile animal models suggest that ART procedures alone can induce fetal and placental growth changes and that these changes can result in different outcomes dependent on culture conditions, genetic background, and stage of embryo transfer, among other variables (Bloise et al., 2014). In mouse, IVF concepti display placental overgrowth and small- and large-for-gestational age offspring, outcomes also observed in some human IVF pregnancies (Bloise et al., 2012; Chen et al., 2015a; Chen et al., 2015c; Collier et al., 2009; de Waal et al., 2015a; Sui et al., 2014; Tan et al., 2016)." (lines 62-68)

We do want to mention that several studies, particularly earlier studies using different conditions that are now considered suboptimal (high hormone levels, suboptimal culture media, suboptimal or undescribed oxygen levels during embryo culture, etc.) are excluded as the relevancy to current recommended human IVF standards is questionable. If there are papers in addition to those that have already been added, please provide these references because we would include them.

We agree with this reviewer that we missed the opportunity to describe why outcomes, especially with regard to fetal weight differences at E12.5, are different from previous studies. We have added additional discussion.
Added text: "This outcome is different from previous studies, which have predominantly compared IVF concepti to S or ET controls, rather than Natural controls. The inclusion of all groups for comparison in this study aids in determining how ET and S still contribute a portion of effects among the various phenotypes." (lines 285-288).

Second decision letter

MS ID#: DEVELOP/2019/186551

MS TITLE: Assisted reproductive technologies induce temporally-specific placental defects and the preeclampsia risk marker sFLT1 in mouse.

AUTHORS: Lisa A Vrooman, Eric A Rhon-Calderon, Olivia Y Chao, Duy K Nguyen, Laren (Riesche) Narapareddy, Asha K Dahiya, Mary E. Putt, Richard M Schultz, and Marisa S Bartolomei

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments (and the editor's note appended below) in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Editor's note:

Your response to reviewer 2 comments is appropriate.

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Please consider addressing in the Discussion issues of the limitation of the experimental design: the small number of pseudopregnant recipients and asynchrony of embryonic age and gestational age.

Reviewer 1

Advance summary and potential significance to field

The authors have carried out a longitudinal study (E12.5, E14.5, E18.5) of the impact of individual and combined assisted reproduction techniques (ART) on embryos and placentas with the comparator being pregnancies in naturally cycling mice. These are very challenging experiments. Embryos cultured in vitro were the most affected (as compared to the ET and superovulated groups) and showed both early (E12.5) effects on embryo weights and placental abnormalities as well as later (E18.5) effects on embryo weight, placental overgrowth (with region-specific effects) and increases in sFLT-1 (a protein implicated in preeclampsia in humans). The longitudinal placental histology and marker studies are particularly interesting

Comments for the author

I have reviewed the revised manuscript and changes made to the figures, supplementary information and text. The authors have responded to my concerns and comments. In particular, the inclusion of new Figs. S2 and S9 allows a fuller appreciation of the data.

Minor modification:
-In relation to changes to the text in response to Comment 4 (lines 137-140- revised version), I suggest that reference to E18.5 be added back to the sentence: e.g. “These weight trends….were no longer statistically different from natural controls at E18.5”

Reviewer 3

Advance summary and potential significance to field

As previous review

Comments for the author

The authors have addressed most of the concerns raised in the previous review. In particular, the statistical analysis is improved by including dam as a covariate. It is a limitation that dam was not utilised as subject, but I accept that in this case manipulations were to embryos not dams. The limitation of small recipient dams numbers should be noted in the Discussion.

An important remaining issue is that of recipient pseudopregnancy stage and its impact on fetal weight in later gestation, and the comparison to the naturally mated control group. The authors have acknowledged in their rebuttal that their designation of gestation date / developmental stage is with regard to embryo developmental stage, not day of tract development at transfer. It is well known that tract receptivity is a limiting factor in implantation, such that blastocyst stage embryos transferred into day 2.5 tracts will implant 24-48 h later, unlike blastocysts in natural conception cycles that implant day 3.5-day 4.0 (within 12 h of blastulation). There are good studies that demonstrate both tract and embryo are determinants of the timing of implantation in mice, that could be mentioned (Ueda et al. Biol Reprod 2003, PMID 12773412; Li et al, Sci Reports, PMID 26531680). The rationale that a similar lack of synchrony occurs in humans is not correct - any scale of impact of 1-2 days of difference in phase between embryo and tract development, adjusted for gestation length, is much smaller in humans. Conclusions about the impact of treatments on fetal weight (abstract, discussion) should be modulated accordingly. The second paragraph in the discussion (around line 228) should include recognition of the prospect that embryo implantation has occurred later in all the embryo transfer groups, and this likely contributes to reduced fetal weight in mid-gestation after embryo transfer. However I agree with the authors that this doesn't account for the placental effects reported, especially for parameters where ET and EC groups are

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different, but unless mentioned as a contributing factor, the work may be dismissed because of this limitation.

Additionally, there are several minor points that could be addressed:

1. The significance of the phrase ‘temporally-specific‘ is unclear, so can probably be removed.

2. The summary statement is an over-statement, as the work is in mice not human. Perhaps ‘Here, we investigate…’ is more accurate.

3. Line 38: The phrase ‘to control for the effects of individual procedures...’ would be more accurate as ‘to investigate the effects of individual procedures…’.

4. Line 40-41: ‘embryo transfer’ would be better as ‘transfer of in vivo-developed embryos’, and ‘embryos cultured in vitro’ would be better as ‘embryos cultured in vitro before transfer’.

5. The authors are missing a critical reference for impact of IVF on human singleton pregnancies: Marino et al. 2014 Plos One PMID: 24416127.

6. Line 105: It would be helpful to add the word ‘transferred’ to read ‘transferred culture embryos’

7. Line 108: remove the word ‘dramatic’

8. Line 450: were super-ovulated CF1 mice (IVF, EC and S groups) pre-pubertal, or adult? The age of each group of females at the time of experimental use should be given.

9. Line 468: should be ‘defined as E3.5’

10. Line 468: the minimum number of pregnant recipient dams per group should be given here, as well as the minimum number of concepti.

11. Line 529: It is disappointing that a correct negative control (rabbit non-immune serum) was not conducted in IHC experiments. However, that robust staining with the expected staining patterns was achieved, provides a reasonable indication that antigen-specific detection was achieved.

12. Line 571: Why is it that intensity of glycogen staining was taken as an important parameter? Surely the key factor is area of positive glycogen staining, if staining is intended to define placental glycogen cells? This should be clarified.

13. Line 610: It should be specified that the ELISA is specific for mouse sVEGFR1/sFLT

Second revision

Author response to reviewers' comments

We thank the Editor and reviewers again for their very careful attention to our manuscript. We have addressed all of their comments, which has enhanced the clarity of the manuscript.

Reviewers’ Comments

Editor’s note:

Your response to reviewer 2 comments is appropriate.

Please consider addressing in the Discussion issues of the limitation of the experimental design: the small number of pseudopregnant recipients and asynchrony of embryonic age and gestational age.

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We have added limitation of number of pseudopregnant recipients as well as addressed concerns about asynchrony of embryonic age created by embryo transfer in the Discussion section (detailed below). We have addressed all other concerns/comments with the newly added text highlighted in green in our detailed responses below as well as in the manuscript document.

Reviewer 1 Advance summary and potential significance to field
The authors have carried out a longitudinal study (E12.5, E14.5, E18.5) of the impact of individual and combined assisted reproduction techniques (ART) on embryos and placentas with the comparator being pregnancies in naturally cycling mice. These are very challenging experiments. Embryos cultured in vitro were the most affected (as compared to the ET and superovulated groups) and showed both early (E12.5) effects on embryo weights and placental abnormalities as well as later (E18.5) effects on embryo weight, placental overgrowth (with region-specific effects) and increases in sFLT-1 (a protein implicated in preeclampsia in humans). The longitudinal placental histology and marker studies are particularly interesting

Reviewer 1 Comments for the author
I have reviewed the revised manuscript and changes made to the figures, supplementary information and text. The authors have responded to my concerns and comments. In particular, the inclusion of new Figs. S2 and S9 allows a fuller appreciation of the data.

Minor modification:
-In relation to changes to the text in response to Comment 4 (lines 137-140- revised version), I suggest that reference to E18.5 be added back to the sentence: e.g. “These weight trends….were no longer statistically different from natural controls at E18.5”
We have taken this suggestion and clarified the sentence by adding back ‘at E18.5’ to the sentence (lines 140-141).

Reviewer 3 Advance summary and potential significance to field
As previous review

Reviewer 3 Comments for the author
The authors have addressed most of the concerns raised in the previous review. In particular, the statistical analysis is improved by including dam as a covariate. It is a limitation that dam was not utilised as subject, but I accept that in this case manipulations were to embryos not dams. The limitation of small recipient dams numbers should be noted in the Discussion.
We have added this caveat to the second sentence of the Discussion, which now reads, ‘Although performed on a small number of total dams per group to accommodate the multiple time points and experimental groups, to our knowledge, this is the first experimental study to demonstrate that ART procedures independent of factors related to underlying infertility lead to placental vasculature defects and increased sFlt1, cellular phenotypes relevant to preeclampsia in humans.’ (lines 272-276).

An important remaining issue is that of recipient pseudopregnancy stage and its impact on fetal weight in later gestation, and the comparison to the naturally mated control group. The authors have acknowledged in their rebuttal that their designation of gestation date / developmental stage is with regard to embryo developmental stage, not day of tract development at transfer. It is well known that tract receptivity is a limiting factor in implantation, such that blastocyst stage embryos transferred into day 2.5 tracts will implant 24-48 h later, unlike blastocysts in natural conception cycles that implant day 3.5-day 4.0 (within 12 h of blastulation). There are good studies that demonstrate both tract and embryo are determinants of the timing of implantation in mice, that could be mentioned (Ueda et al. Biol Reprod 2003, PMID 12773412; Li et al, Sci Reports, PMID 26531680). The rationale that a similar lack of synchrony occurs in humans is not correct - any scale of impact of 1-2 days of difference in phase between embryo and tract development, adjusted for gestation length, is much smaller in humans. Conclusions about the impact of treatments on fetal weight (abstract, discussion) should be modulated accordingly. The second paragraph in the discussion (around line 228) should include recognition of the prospect that embryo implantation has occurred later in all the embryo transfer groups, and this likely contributes to reduced fetal weight in mid-gestation after embryo transfer. However I agree with the authors that this doesn't account for the placental effects reported, especially for parameters where ET and EC groups are

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different, but unless mentioned as a contributing factor, the work may be dismissed because of this limitation.

We thank the reviewer for their suggestions and have tried to address the overall issue. We want to note that the references specified by this reviewer concerning timing of embryo transfer are important, but that these references pertained to when blastocysts (3.5 dpc) were transferred into the oviducts of a 0 or 1 dpc pseudopregnant female. This is both a larger time discrepancy than in our study and also exposes blastocysts to a different anatomical/tract environment than in vivo. We want to assure the reviewer that the gold standard for blastocyst transfer is transfer into the uterus of 2.5 dpc pseudopregnant females and this is considered more optimal that 3.5 dpc because manipulated embryos have time to catch up developmentally (Nagy et al., 2003). We have added this detail to the Methods (lines 484-486). Personal communication with Paratechs, the manufacturer of the NSET device used, also commented that 2.5 dpc pseudopregnant females also ensure that embryos do not miss the window of implantation, which closes by the end of 4 dpc.

We do not believe the newly edited Abstract sentence concerning embryo transfer results are overstated especially as it is related to the phenotype over time, the point being that differences in fetal weight with ET are significantly different at E12.5 but later in development they are not. ‘We demonstrated that transfer of blastocysts conceived without hormone stimulation and in vivo preimplantation development can impair early placentation and fetal growth, but this effect normalizes by term.’ (lines 39-42).

In the Discussion, we have added details of the limitation of comparing Natural and ET groups, as well as a new reference that has directly compared these two groups that we think would be helpful for the readers’ understanding. It also speaks to the Reviewer’s concern that ‘embryo implantation has occurred later in all the embryo transfer groups’, and that ‘this likely contributes to reduced fetal weight in mid-gestation after embryo transfer’, as there is no evidence of this in the referenced study.

New text: ‘However, there is a caveat to the comparison to Natural concepti because inability to control both the timing of implantation and overall litter size, subsequently impacting fetal weight and placentation results.’ (lines 291-294).

New text: This outcome is different from a recent paper by Menelaou et al, comparing E10.5 ET and Natural concepti (Menelaou et al., 2020). They report significantly smaller ET placentas compared to Naturals with no differences in fetal weight. Together with E10.5 placental transcriptomic analysis, they concluded that embryo transfer significantly impacts placental transcriptome and growth, with signs toward adaptive response that would improve placental function. This observation supports that fetal weights early in gestation are unaffected by factors related to embryo transfer. We postulate that by E12.5, as fetal growth increases, fetal weight differences may become more apparent-- although we cannot discount differences in mouse strain and embryo transfer procedures to explain the discrepancy between the studies. Their overall conclusion that ET placentas mount an adaptive response, is aligned with our results. (lines 297-308).

Additionally, there are several minor points that could be addressed:

1. The significance of the phrase ‘temporally-specific‘ is unclear, so can probably be removed.
We appreciate the suggestion and agree that ‘temporally-specific’ is vague, but this is due to character limit constraints. The phrase reflects that the observed placental defects are specific to different stages of placental development and also distinguishes it from previous studies examining a single time point.

2. The summary statement is an over-statement, as the work is in mice not human. Perhaps ‘Here, we investigate…’ is more accurate.
In accordance with the Reviewer’s suggestion, we have revised the word ‘show’ to ‘investigate’. The edited sentence now reads, ‘Here, we investigate how individual procedures contribute to placental defects across development using a mouse model.’ (lines 32-33).

3. Line 38: The phrase ‘to control for the effects of individual procedures...’ would be more accurate as ‘to investigate the effects of individual procedures…’.

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We have changed ‘control for’ to ‘investigate’. The edited sentence now reads, ‘To elucidate their underlying causes, we conducted a longitudinal analysis of placental development and fetal growth using a mouse model to investigate for the effects of individual ART procedures, namely, hormone stimulation, in vitro fertilization (IVF), embryo culture, and embryo transfer.’ (lines 36-39).

4. Line 40-41: ‘embryo transfer’ would be better as ‘transfer of in vivo-developed embryos’, and ‘embryos cultured in vitro’ would be better as ‘embryos cultured in vitro before transfer’.
In accordance with the Reviewer’s suggestion, we have revised the sentence for clarity, which now reads, ‘We demonstrated that transfer of blastocysts naturally conceived without hormone stimulation and that developed in vivo prior to transfer can impair early placentation and fetal growth, but this effect normalizes by term. In contrast, embryos cultured in vitro before transfer do not exhibit this compensation but rather display placental overgrowth, reduced fetal weight, reduced placental DNA methylation, and increased sFLT-1, an anti-angiogenic protein implicated in causing the maternal symptoms of preeclampsia in humans.’ (lines 39-45).

5. The authors are missing a critical reference for impact of IVF on human singleton pregnancies: Marino et al. 2014 Plos One PMID: 24416127.
We thank the reviewer for bringing this reference to our attention. It has been added to lines (56-61), which now reads, ‘Studies of human singleton pregnancies demonstrate that ART is associated with abnormal placentation, preeclampsia, small- and large-for-gestational age babies, preterm birth, miscarriage, perinatal mortality, pregnancy complications, and congenital disorders (Daniel et al., 1999; Haavaldsen et al., 2012; Luke et al., 2017; Luke et al., 2019; Marino et al., 2014; Pandey et al., 2012; Schieve et al., 2007; Stern et al., 2018; Wisborg et al., 2010).’

6. Line 105: It would be helpful to add the word ‘transferred’ to read ‘transferred culture embryos’
We have added the word ‘transferred’. The edited sentence now reads, ‘Concepti derived from transferred cultured embryos failed to achieve normal fetal weight by term and were found to have placental abnormalities reflective of placental overgrowth and preeclampsia—abnormalities observed in human ART pregnancies.’ (lines 106-109).

7. Line 108: remove the word ‘dramatic’
We have removed the word ‘dramatic’. The edited sentence now reads, ‘Importantly, all these phenotypes were linked to loss of placental DNA methylation which suggests underlying epigenetic perturbations incurred during embryo culture cause late gestational placental abnormalities and impair placental compensatory mechanisms.’ (lines 109-112).

8. Line 450: were super-ovulated CF1 mice (IVF, EC and S groups) pre-pubertal, or adult? The age of each group of females at the time of experimental use should be given.
We have clarified this detail in the Methods section line 459: ‘All CF-1 female mice were sexually mature and utilized between 2-3 months of age.’

9. Line 468: should be ‘defined as E3.5’
We thank the reviewer for finding this typo. We have changed ‘at’ to ‘as’. The edited sentence now reads, ‘Day of blastocyst transfer was defined as E3.5 and concepti were collected at E12.5, 14.5, and 18.5 (+9.0, 11.0, 15.0 days after transfer). (line 487).

10. Line 468: the minimum number of pregnant recipient dams per group should be given here, as well as the minimum number of concepti.
This information can be found in the Tissue Collection section (lines 503-508), where we explain the number of concepti analyzed, rather than in the Generation of Natural, ET, S, EC, and IVF concepti section which describes the preimplantation procedures. Detailed information is also found in Supplemental Figure 2 and in each Figure legend.

11. Line 529: It is disappointing that a correct negative control (rabbit non-immune serum) was not conducted in IHC experiments. However, that robust staining with the expected staining patterns was achieved, provides a reasonable indication that antigen-specific detection was achieved.
We appreciate the Reviewer’s comment.

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12. Line 571: Why is it that intensity of glycogen staining was taken as an important parameter? Surely the key factor is area of positive glycogen staining, if staining is intended to define placental glycogen cells? This should be clarified.
To clarify, the PAS staining is not intended to define placental glycogen cells as glycogen cell area as a proportion of the junctional zone was already measured by ISH experiments in Figure 6. The purpose of measuring the intensity of PAS staining was to determine relative glycogen content to determine if glycogen stores within the glycogen cells were normally utilized or abnormally retained. Accordingly, we have revised this section of the Results for clarity.
The revised sentences now reads, 'Because glycogen cells are known to deplete their glycogen stores prior to term (Coan et al., 2006) we determined by Periodic acid-Schiff staining whether glycogen cell content was abnormally retained in IVF placentas in a subset of E18.5 IVF and control placentas. Despite differences in the total amount and proportional contribution of glycogen cells to the junctional zone at E18.5, IVF placental glycogen content relative to glycogen cell area was not significantly different from controls, suggesting that placental glycogen stores were normally utilized prior to term (Fig. S7).' (lines 204-211).

13. Line 610: It should be specified that the ELISA is specific for mouse sVEGFR1/sFLT
We have added the word 'Mouse'. The edited sentence now reads, 'Serum was diluted 1:25 and ELISA was performed in duplicate by individuals blinded to experimental group using Mouse sVEGFR1/FLT-1 DuoSet ELISA and DuoSet ELISA Ancillary Reagent Kit 2 (catalog # DY471, #DY008, R&D Systems, Minneapolis, MN) according to manufacturer's instructions.' (line 629-632).

Third decision letter

MS ID#: DEVELOP/2019/186551

MS TITLE: Assisted reproductive technologies induce temporally-specific placental defects and the preeclampsia risk marker sFLT1 in mouse.

AUTHORS: Lisa A Vrooman, Eric A Rhon-Calderon, Olivia Y Chao, Duy K Nguyen, Laren (Riesche) Narapareddy, Asha K Dahiya, Mary E. Putt, Richard M Schultz, and Marisa S Bartolomei
ARTICLE TYPE: Research Article

I am satisfied with your response and the last revision. This manuscript has been accepted for publication in Development, pending our standard ethics checks.

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