Dysregulation of integrin αvβ3 and α5β1 impedes migration of placental endothelial cells in fetal growth restriction

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Original submission

First decision letter

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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 Reviewer 1 has 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, 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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.

Reviewer 1

Advance summary and potential significance to field

In this study, Gumina et al. describe a novel mechanism for placental endothelial dysfunction leading to fetal growth restriction (FGR), namely dysregulated interaction between integrin α5β1 and αvβ3 adhesion complexes and fibronectin. This interaction is key for cell migration and is proposed to be implicated in poor angiogenesis in FGR placentae. Although the proposed mechanism is novel, and the work itself includes several clever experiments, several critical limitations significantly reduce the ability to interpret their results in the context of human pregnancy.

Comments for the author

In my opinion, the main limitations are the following:
1. The sample size is small. I perfectly understand that collecting samples from patients that fulfill a specific set of criteria is not an easy task. However, while the patients are matched for some criteria (listed in Table 1), there are many other confounding factors that could contribute to the observed phenotype. Examples of such cofounding factors include maternal smoking status, maternal BMI, alcohol consumption, ethnic background etc. With n=6 samples per group, and even fewer biological replicates for the experiments shown in Figures 4, 5 and 6, the confidence for the reproducibility of these findings in an independent cohort of samples is in my view small (see the additional comments related to statistical analysis at point 4).
2. One of the key differences between the two groups of samples is the gestational age (mean of 39 weeks for controls and 29 weeks for FGR). The FGR group is also more heterogeneous in terms of gestational ages (24-34 weeks). I do understand that the termination of pregnancy for the FGR group at earlier stages is a necessity. However, how can the authors be confident that what they measure in all the experiments does not simply reflect the significant difference in the gestational stage between the two sets of samples (an average of ten weeks)? Control preterm placenta collected from preterm pregnancies with appropriately grown fetuses have been used in previous studies (PMID: 22264586). Ideally, the authors should strive to match the samples for this very important parameter.
3. I do not understand the choice for the use of feto-placental endothelial cells of macrovascular origin in this study. The formation of the placental vascular network is mainly attributable to feto-placental endothelial cells of microvascular origin (reviewed in PMID: 21215450) and there are many differences in terms of phenotype, gene expression patterns and functional characteristics between human feto-placental endothelial cells of micro- and macrovascular origin (see for example PMID: 20587779 and PMID: 30269024). Protocols to isolate microvascular feto-placental endothelial cells are available through publications (PMID: 22264586, PMID: 30269024). At the very least, this limitation, and the ones highlighted above should be presented with honesty in the discussion.
4. Although the authors provide a brief description in the methods for the statistical tests used, I think they need to add in all figures legends details for the specific test used in each panel. Some of the p values presented suggest to me the choice of a wrong statistical test. For example, in Figure 4B the graph depicting “Active αvβ3/total αv” has three biological replicates, each one has 3 technical replicates. The correct statistical test in this case is a Mann-Whitney test, because there are only n=3 samples per group. Based on the spread of the three average values for each group shown in this figure, I think that the Mann-Whitney test should be either non-significant or, at the very best, have a very marginally significant p value, and not p=0.007. Same issue applies to many other graphs presented in this study.

Minor points:
1. There is a typo at line 66: “weight less is less than the 10th percentile…”
2. There is conflicting information given on the passage of the cells used in this study: “All cellular experiments used cells between the second and fourth passage” (lines 487-488) versus “only primary cells up to fifth passage were used” (line 381). They also say: “cells were tested for contamination every 3 months” (lines 382-383). Are these cells in culture for months?
3. Why are the protein loads used for western blotting so variable? The total protein loaded in each well can be measured prior to the western blot. It would be easier to appreciate visually any

differences in protein expression in westerns with more uniform loading. The line separating the two groups of samples in Figure 3B (ITGα5) is misplaced.

Reviewer 2

Advance summary and potential significance to field

Demonstration of abnormal motility of endothelial cells in cases of fetal growth restriction and investigation into the underlying integrin mechanisms involved.
Represents a a novel mechanism that contributes to the angiogenic deficiencies in the FGR placenta.

Comments for the author

In the discussion the authors should address what impact altered endothelial motility would have on the vasculogenic and angiogenic disturbances seen in FGR

First revision

Author response to reviewers' comments

Thank you for your insightful comments, which have helped us improve this manuscript, and for the opportunity to provide a revision. We respectfully resubmit this manuscript entitled "Dysregulation of integrin αvβ3 and α5β1 impedes migration of placental endothelial cells in fetal growth restriction" for reconsideration. Responses to specific comments are listed below.

Reviewer #1:
1. The sample size is small. While patients are matched for some criteria (listed in Table 1), there are many other confounding factors that could contribute to the observed phenotype. Examples of such confounding factors include maternal smoking status, maternal BMI, alcohol consumption, ethnic background, etc. With n=6 per samples per group, and even fewer biological replicates for the experiments shown in Figures 4, 5, and 6, the confidence for the reproducibility of these findings in an independent cohort of samples is in my view small.

- We appreciate this comment and agree that clinical confounders are important considerations for human samples. We attempted to control for as many clinical variables as possible, and all individuals who had a history of tobacco, alcohol, cannabis, or illicit drug use during pregnancy were ineligible for recruitment.
- To more clearly describe our cohorts, we have added earliest pregnancy BMI (we were unable to obtain pre-pregnancy BMI within one year of gestation for a few subjects), which was not significantly different between control and FGR cohorts. Although the absolute number of nulliparous subjects varied between the two groups, these were not significantly different as determined by Fisher's exact test. None of the subjects had a conception as a result of in vitro fertilization, which could also act as a confounder.
- With regard to maternal race/ethnicity, 5/6 subjects in each cohort were of White or undisclosed race/ethnicity, with one subject in the control group being of Latina (non-white) ethnicity and one subject in the FGR cohort being of Asian descent. We absolutely acknowledge that race/ethnicity are important clinical factors. However, we unfortunately were unable to perfectly control for this as the number of eligible subjects were limited for two main reasons. First, severe, early-onset FGR only accounts for ~2- 3% of the overall population. Second, our exclusion criteria were stringent in order to attempt to control for confounders as best as possible, further limiting eligible cases.
- We address the concern regarding biological replicates in point #4.

2. One of the key differences between the two groups is the gestational age (mean of 39 weeks for controls and 29 weeks for FGR). The FGR group is also more heterogeneous in terms of gestational ages (24-34 weeks). How can the authors be confident that what they measure in all the

experiments does not simply reflect the significant difference in the gestational stage between the two sets of samples? Control preterm placental collected from preterm pregnancies with appropriately grown fetuses have been used in previously studies. Ideally, the authors should strive to match the samples for this very important parameter.

- Thank you for this important comment. We acknowledge that angiogenesis is not static throughout gestation, and the lack of preterm placentas is a limitation in our study. However, utilizing placentas from appropriately grown, gestational age-matched fetuses that delivered preterm for some other etiology other than FGR also leads to other potential confounders. For instance, there is increasing data to suggest that placental pathology related to placental insufficiency may also be evident in many cases of spontaneous preterm birth of appropriately grown fetuses (PMID 25124429, 34360662, 35246973, 31665946, 23130816). Furthermore, our previously published data suggest that gestational age does not significantly affect migratory properties of isolated placental endothelial cells, where endothelial cells isolated from preterm, gestational age-matched, appropriately grown fetuses exhibit similar motility as term control endothelial cells.
- We have added this to our discussion.

3. I do not understand the choice for the use of fetoplacental endothelial cells of macrovascular origin in this study. The formation of the placental vascular network is mainly attributable to fetoplacental endothelial cells of microvascular origin (reviewed in PMID: 21215450), and there are many differences in terms of phenotype, gene expression patterns, and functional characteristics between human fetoplacental endothelial cells of micro- and macrovascular origin (see for example PMID: 20587779 and PMID: 30269024). At the very least, this limitation, and the ones highlighted above, should be presented with honesty in the discussion.

- Thank you for bringing up this point. We absolutely agree that there are differences between micro- and macrovascular endothelial cells of the placenta and that further discussion surrounding these nuances are needed. This has been incorporated into the Discussion.
- We chose to utilize macrovascular placental endothelial cells isolated from the chorionic plate for several reasons. First, the method in which our lab isolates *microvascular* placental endothelial cells (mincing/digestion/Percoll/several selections with CD31) requires multiple passages to obtain a pure population, leading to higher passage numbers to start. In contrast, we are able to obtain a pure macrovascular population within the first passage and to thus be able to use lower passage number cells. Second, by virtue of our isolation process, our macrovascular endothelial cells are purely limited to the chorionic plate vessels, ensuring a pure population. It is possible that several of the previously published methods for microvascular cell isolation, especially those that leverage cannulation of vessels, include some macrovascular endothelial cells from the chorionic plate and/or stem villi, as examples. Third, our lab had previously established that macrovascular endothelial cells exhibit migratory defects, suggesting that they display angiogenic dysfunction. Fourth, proteomic analyses of control versus FGR HUVECs by another group (PMID: 27208404) has shown that several proteins implicated in angiogenesis are altered in these macrovascular endothelial cells.
- Ultimately, we acknowledge that it remains unknown whether a dysfunctional macrovasculature drives additional impairments in the microvasculature or whether this model best recapitulates placental angiogenesis. However, for the reasons noted above, we felt it was important to start with these well-characterized, macrovascular endothelial cells. In future studies, we will investigate whether these altered mechanisms are similar in the microvasculature.
- This has been added to the discussion.

4. Although the authors provide a brief description in the methods for the statistical tests used, I think they need to add in all figures legend details for the specific test used in each panel. Some of the p values presented suggest to me that the choice of a wrong statistical test. For example, in Figure 4B, the graph depicting “Active αvß3/total αv” has three biological replicates, with each one having 3 technical replicates. The correct statistical test in this case is a Mann-Whitney test, because there are only n-3 samples per group. Based on the spread of the three average values for

each group shown in this figure, I think that the Mann-Whitney test should be either non-significant or, at the very best, have a very marginally significant p value, and not p=0.007. Same issue applies to many other graphs presented in this study.

- We agree with the reviewer that fewer subject numbers were included in the original Figures 4 and 6. Thus, we have performed additional experiments to increase the biological and technical replicates, which is now reflected in the Results section and figures. All figure legends have also been updated as requested with the specific statistical test used for each figure.
- For the updated Figure 4, we have opted to perform a biochemical assay that compares the active forms bound to substrate between FGR and control subjects. In contrast to TIRF microscopy, which was limited to a few cells captured with a 100x objective, we feel that this approach gives a better view of the bigger picture as it is assessing focal adhesion complexes isolated from 6 million cells per subject.
- For Figures 5 and 6, we appreciate that a biological n of 6 in each group may seem like a small number, but we are actually analyzing the number of focal adhesions and vesicles per cell. Other groups investigating focal adhesions report the number of adhesions analyzed (PMID: 32234213), which range from approximately 300-2500 adhesions, or they also report regions of interest (ROI) (PMID: 31628312) with 15-30 ROIs depending on the analysis. In comparison, in Figure 5, we are analyzing between 1200-6000 adhesions per subject (depending on the antibody stain), and from 8-40 ROIs per subject. For Figure 6, we are analyzing 72 ROIs per subject per stain. Given these values, we feel that our work is on par with the field. The figure legends have been revised to reflect this information.

Minor points:

1. There is a typo at line 66: “weight less is less than the 10^{th} percentile…”
   - We apologize for our oversight. This has been corrected.

2. There is conflicting information given on the passage of the cells used in this study: “All cellular experiments used cells between the second and fourth passage” (lines 487-488) versus “only primary cells up to the fifth passage were used” (line 381). They also say “cells were tested for contamination every 3 months” (lines 382-383). Are these cells in culture for months?
   - We are sorry for our lack of clarity. Our cells are not in culture for months and are only in culture for a few days at most, which is the timeframe that allows cells to reach an adequate number in order to perform the experiment. We regularly test for contamination.
   - With regard to passage, we have previously shown our cells retain phenotype up to passage 5, but the experiments reported here only used cells between passage 2-4.
   - These points have been clarified in the text.

3. Why are the protein loads used for western blotting so variable? The total protein loaded in each well can be measured prior to the western blot. It would be easier to appreciate visually any differences in protein expression in westerns with more uniform loading. The line separating the two groups of samples in Figure 3B (ITGA5) is misplaced.
   - We calculate total protein with a BCA assay and attempt to load equal quantities. For our immunoblotting experiments, we used an automated, capillary system (Protein Simple Jess). Unlike conventional Western blotting, this format uses very small volumes, which can lead to inadvertent variability in loading. To address this, we used a protein normalization standard that has been shown to be at least equivalent to traditional housekeeping proteins. While the loading may look variable, there were no significant differences in the total protein stain between groups, and this has been added to supplemental data. Figure 3B has also been updated.

Reviewer #2:
1. In the discussion, the authors should address what impact altered endothelial motility would have on the vasculogenic and angiogenic disturbances seen in FGR.
- Thank you for your insight. We have elaborated on this in the Discussion.

Second decision letter

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The additional experimental work and the changes made to the discussion have improved considerably this paper. Although there are some important limitations in this study, these have been discussed appropriately and the readers can make their own mind as to the significance of these findings.

Comments for the author

I do not have any additional suggestions.