Cytotrophoblast extracellular vesicles enhance decidual cell secretion of immune modulators via TNF-α

Sara K. Taylor, Sahar Houshdaran, Joshua F. Robinson, Matthew J. Gormley, Elaine Y. Kwan, Mirhan Kapidzic, Birgit Schilling, Linda C. Giudice and Susan J. Fisher
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Original submission

First decision letter

MS ID#: DEVELOP/2019/187013

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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 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, 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.

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.

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

Taylor et al reported the characterisation of placental small EVs, isolated as the 100,000 g fraction from a culture of second trimester human cytotrophoblast. They characterized EV morphology, and several protein markers. A mass spectrometry screen showed proteins that are involved in numerous important processes. Using Luminex-based cytokine array they found enrichment for many immune factors, including TNFa. Functionally, they showed that these 100,000 g EVs, when applied to decidual stromal fibroblasts, stimulate the production of mRNAs and proteins, including targets for NF-kB, such as IL-8, which was inhibited by the addition of a soluble form of TNF receptor.

The data are interesting, and a large number of experiments are presented. The high-throughput data add to our knowledge of small EVs produced by human trophoblasts.

Comments for the author

1. The 16,500 g precipitate contain a mixture of vesicle types, as identified by the authors. What was the significance of their analysis?
2. Because of the limited materials, the difficulty in performing gradient-based precipitation is understandable, yet this raises questions about purity, and therefore the equality of the results. For example, the authors show that the 100,000 g EVs are enriched for fibronectin. Fibronectin is known to be a sticky protein, and a number of papers, most prominently the landmark paper by Jeppesen et al., (Cell 2019; 177:428–445) showed that fibronectin is not a part of small EV cargo.
3. Along the same line, to ensure that the functional data (immune response) from target decidual stromal fibroblasts indeed reflect a response to EVs, the authors should have included controls where the EV-depleted medium was devoid of the observed effect.
4. To validate the specificity of decidual stromal fibroblast response, the effect of trophoblast EVs on fibroblasts that were not obtained from the decidua, serving as a negative control, should have been tested.
5. The introduction is lengthy, and includes questionable statements of unclear relevance. The results section also contain many speculative statements that may belong to the Discussion.

Reviewer 2

Advance summary and potential significance to field

The authors in this manuscript studied extracellular vesicles (EVs) secreted from cytotrophoblasts (CTBs) from 2nd trimester human placentas. A fraction of EVs separated by differential ultracentrifugation mainly consists of exosomes. Using mass spectrometry, they profiled exosomes and other EVs from cytotrophoblasts, and identified an array of cytokines in exosomes including TNF-α. These cytokines in turn stimulate inflammatory responses in maternal decidual cells, which may be critical for pregnancy outcomes.
Although numerous studies suggest that exosomes produced from placental cells modulate maternal immune tolerance of fetuses, most studies focused on exosomes derived from syncytiotrophoblasts (STBs). The current study analyzed the components of exosomes produced by CTBs, which extends our understanding of placental derived exosomes. The global profiling of different fractions of EVs by CTBs provides an important library for future studies.
However, physiological roles of exosomes secreted by CTBs need be elaborated. For example, some invasive CTBs have direct contact with maternal decidual tissues. Intercellular interaction could be executed by direct cell-cell contacts or paracrine secretions. This raises the question of the role of exosomes in influencing fetal functions. This issue needs to be discussed in the manuscript to strengthen the concept of exomes influencing the maternal physiology and that of fetal well-being.

They also need to discuss the abundance of exosomes produced by STBs vs CTBs in pregnancy and the differences in components in STB vs CTB exosomes.
In conclusion, this is a solid work showing Mass spec analysis of components of derived from CTBs. With appropriate discussion, this study will provide meaningful contribution of exosome in human pregnancy biology.

Comments for the author

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In conclusion, this is a solid work showing Mass spec analysis of components of derived from CTBs. With appropriate discussion, this study will provide meaningful contribution of exosome in human pregnancy biology.

First revision

Author response to reviewers' comments

We thank the reviewers for the constructive review of our manuscript. Their comments are stated below in roman font and our responses follow in italic font. Changes to the manuscript are in bold font. Parts of the Discussion and the Results Sections that were eliminated appear as crossed out text.

Reviewer #1

1. The16,500 g precipitate contains a mixture of vesicle types, as identified by the authors. What was the significance of their analysis?

We were interested in the contents and functions that were specific to, or enriched in, the 100,000 xg vesicle fraction isolated from human placental cytotrophoblasts. We used two comparators. One was the 100,000 xg vesicle fraction from the K562 erythroleukemic cell line. The other was the heterogeneous mixture of vesicles that the16,500 xg pellet contained. We wanted to include this fraction because we knew from preliminary experiments that it included an unusual population of very large extracellular vesicles that are likely ectosomes, which are formed by outward budding of the plasma membrane. Human placental cytotrophoblasts produce sizeable quantities of these vesicles. Release of the largest subpopulation can be visualized by videomicroscopy. After the initial characterization, which was part of the submitted manuscript, we also plan to study the functions of the 16,500 xg fraction, which the data presented in this paper suggests could, in some instances, be distinct from the 100,000 xg vesicles.

2. Because of the limited materials, the difficulty in performing gradient-based precipitation is understandable, yet this raises questions about purity, and therefore the equality of the results. For example, the authors show that the 100,000 g EVs are enriched for fibronectin. Fibronectin is known to be a sticky protein, and a number of papers, most prominently the landmark paper by Jeppesen et al., (Cell 2019; 177:428–445) showed that fibronectin is not a part of small EV cargo.

Our mass spectrometry data showed that several integrin subunits were identified in the 100,000 xg vesicle fraction. This included α5 and α1, which together comprise the major fibronectin receptor. Our work over many years (e.g., (Damsky et al., 1992) has shown that human placental cytotrophoblasts also produce very significant amounts of fibronectin. We surmise that α5/α1, which is a component of the extracellular vesicle membrane complexes with fibronectin, which is a component of the peri-cellular extracellular matrix that surrounds the cells from which they are released.

3. Along the same line, to ensure that the functional data (immune response) from target decidual stromal fibroblasts indeed reflect a response to EVs, the authors should have included controls where the EV-depleted medium was devoid of the observed effect.

We believe the reviewer is asking if the observed effects were specific to EVs or could be attributed to factors in the 100,000 xg vesicle fraction. To isolate this fraction, we performed consecutive ultracentrifugation steps. After the first step, the pellet was washed with PBS and resuspended in this buffer before a high-speed pellet was isolated for a second time. Therefore, we think that at most trace amounts of the conditioned medium, if any, were left in the 100,000 xg extracellular vesicle fraction.

As to our expectations for parallel analyses of the conditioned medium devoid of extracellular vesicles, we know that human placental cytotrophoblasts are an abundant source of secreted TNFα. Therefore, the conditioned medium devoid of extracellular vesicles should induce elements of the cytokine response that was observed with the 100,000 xg fraction.

4. To validate the specificity of decidual stromal fibroblast response, the effect of trophoblast EVs on fibroblasts that were not obtained from the decidua, serving as a negative control, should have been tested.

To clarify, we do not think that the ability to respond to TNFα is unique to decidual cells. In humans, the TNF superfamily consists of 19 ligands and 29 receptors (Vanamee and Faustman, 2018). A search of GTEx and the Human Protein Atlas shows that superfamily members are expressed in various combinations by nearly every cell type that has been profiled, including fibroblasts. Furthermore, a few of the ligands bind to a single receptor, but most interact with more than one. In turn, particular ligand-receptor combinations signal via different pathways (e.g., MAPKs, NFκB or AKT; (Kalliolias and Ivashkiv, 2016). Given the relatively new appreciation of fibroblast diversity in seemingly homogenous tissue and organ populations (Lynch and Watt, 2018), we did not think it would be possible to find a negative control fibroblast population that would not have some element of the TNFα responses we observed.

However, our previous work did address the unique ability of uterine stromal cells to decidualize (Aghajanova et al., 2010). Specifically, we attempted to differentiate human neonatal dermal fibroblasts by treating the cells with 8-Br-cAMP and showed them to be nonresponsive as compared to human endometrial stromal cells, which decidualize under the same conditions. Overall the results of this study suggested that other tissue resident fibroblasts are more restricted in their ability to differentiate along this pathway. Other distinctions may include the signaling pathways that are wired to their particular complement of TNFα receptors. We added this concept to the Discussion Section of the paper.

5. The introduction is lengthy and includes questionable statements of unclear relevance. The results section also contains many speculative statements that may belong to the Discussion.

We shortened the introduction by eliminating the history of syncytiotrophoblast extracellular vesicle isolation and characterization, which was summarized in two paragraphs.

We removed the speculation in the Results at the end of the last paragraph of the Section entitled, "Cytotrophoblasts produced extracellular vesicles that contained exosomal and placental markers." The text is copied below:

We immunoblotted for other molecules whose functions in exosomes are relevant to the biology of pregnancy. Placental EVs contained the adhesion-promoting ECM molecule, fibronectin (FN; Fig 2E), whose expression was strikingly higher in 100,000 xg EVs compared to the 16,500 xg fraction. Previous studies showed that the exosomal form of this molecule promotes migration of neighboring cells (Sung et al., 2015, Sung and Weaver, 2017) Exosomal PD-L1 suppresses T cell activation in cancer (Poggio et al., 2019) . As immune evasion is critical for cancer progression and pregnancy success, we asked whether CTB EVs contained this molecule. PD-L1, a suppressor of T cell activation (Poggio et al., 2019) was detected in CTB lysates and both populations of vesicles (Fig. 2F), suggesting that these EVs could have immune inhibitory properties.

We would very much like to keep the discussion points in the next Section entitled, "Global proteomic analysis of CTB EVs." We believe that synthesis of the proteomic results helps the reader to digest this section, which otherwise becomes protein lists.

Reviewer #2

1. …physiological roles of exosomes secreted by CTBs need be elaborated. For example, some invasive CTBs have direct contact with maternal decidual tissues. Intercellular interaction could be executed by direct cell-cell contacts or paracrine secretions. This raises the question of the role of exosomes in influencing fetal functions. This issue needs to be discussed in the manuscript to strengthen the concept of exomes influencing the maternal physiology and that of fetal well-being.

The possible physiological roles of CTB exosomes are now discussed in the bold portion of the first paragraph of the Discussion Section, copied verbatim and underlined below.

Here, we characterize second trimester CTB EVs by TEM, immunoblot, mass spectrometry, and cytokine array. The 100,000 xg fraction was enriched for exosomal markers, and both EV populations were enriched for placental markers, HLA-G and PLAP. We report the discovery of TNF-α in CTB 100,000 xg EVs. These vesicles increased decidual cell transcription and secretion of NF-κB target cytokines: CXCL8/IL-8, IL-6, CCL2, and CXCL1. However, not all targets upregulated by CTB conditioned medium were significantly increased by EVs(Hess et al., 2007). rhTNF-α mimicked the ability of EVs to enhance transcription of NF-κB target genes and secretion of IL-8. A soluble form of the TNF-α receptor inhibited the ability of CTB 100,000 xg EVs to increase dESF secretion of IL-8, linking TNF-α with decidual cell release of this chemokine. These results suggest that CTBs have several ways of communicating with uterine cells, which include direct (e.g., cell-cell contact) and indirect (e.g., soluble factors and EVs) means. If the vesicles are transported in a retrograde manner back to the fetus, they could also have effects, most likely on the vasculature that is in direct contact with the blood in which they would be carried.

2. They also need to discuss the abundance of exosomes produced by STBs vs CTBs in pregnancy and the differences in components in STB vs CTB exosomes.

We added a new paragraph, the eighth of the Discussion Section, that addresses these points.

As compared to STB EVs, those released by CTBs are likely to have different effects. One factor is sheer numbers. STBs form the entire surface of the placenta, estimated to be ~12 m2 at term(Boyd, 1984), making them an abundant source of EVs. In contrast, CTBs, which fuse to form STBs or differentiate into the extravillous subpopulation, are depleted as pregnancy advances. Thus, we believe CTBs likely release, in total, fewer EVs than STBs, especially towards term. Additionally, they have substantially different proteomes. Comparison of the mass spectrometry data for CTB 100,000 xg EVs (Table 2, this paper) and an equivalent STB fraction(Baig et al., 2014)

showed an overlap of ~30%, supporting the theory that there are also substantial functional differences.

The relevant spreadsheet containing these data is appended to this review.

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Second decision letter

MS ID#: DEVELOP/2019/187013

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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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. A s you will see reviewer 1 requests that you insert some additional text in the Discussion regarding the purity of the vesicles. Could you please address this comment in your revised manuscript and in your point-by-point response. If you do not agree with this suggestion explain clearly why this is so. Your manuscript will not require any further review, rather I will look it over prior to acceptance.

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.

Reviewer 1

Advance summary and potential significance to field

This is a re-review. Likely not needed.

Comments for the author

The issue of EV purity remains questionable. The authors responded that their mass spectrometry data showed that several integrin subunits were identified in the 100,000 xg vesicle fraction. This is important, and not surprising, as the presence of other integrins was previously shown by Lyden and others to play a significant role in guiding of exosomes to target tissues (reviewed in PMID: 31063754). Taylor et al also highlighted that their work over the years has shown that human placental cytotrophoblasts produce very significant amounts of fibronectin, and they therefore surmised that the EV integrins are complexed with fibronectin. Because of the isolation technique used by the authors, and in light of work by others (Jeppesen et al., Cell 2019; 177:428–445) that showed that fibronectin is not a part of EV cargo, this needs to be clarified. If experiments cannot be done due to COVID-19, the authors should at least include in the Discussion some qualifications related to the purity of their EV preparations.

Reviewer 2

Advance summary and potential significance to field

This paper characterizes the extracellular exosomes from CTBs in decidual function with respect to immunemodulation by Mass Spec analysis.

Comments for the author

The authors have addressed this reviewer's concerns satisfactorily and strengthened the manuscript.

Second revision

Author response to reviewers' comments

We thank the reviewers and editor for their interest in publishing our work. We restate the remaining comment of Reviewer 1 below along with our answer.

Reviewer #1

The issue of EV purity remains questionable. The authors responded that their mass spectrometry data showed that several integrin subunits were identified in the 100,000 xg vesicle fraction. This is important, and not surprising, as the presence of other integrins was previously shown by Lyden and others to play a significant role in guiding of exosomes to target tissues (reviewed in PMID: 31063754). Taylor et al also highlighted that their work over the years has shown that human placental cytotrophoblasts produce very significant amounts of fibronectin, and they therefore surmised that the EV integrins are complexed with fibronectin. Because of the isolation technique used by the authors, and in light of work by others (Jeppesen et al., Cell 2019; 177:428–445) that showed that fibronectin is not a part of EV cargo, this needs to be clarified. If experiments cannot

be done due to COVID-19, the authors should at least include in the Discussion some qualifications related to the purity of their EV preparations.

Answer

This issue is now addressed in the first paragraph of the Discussion (see bold text in italics), which is repeated below.

Here, we characterized second trimester CTB EVs by TEM, immunoblotting, mass spectrometry, and cytokine array. The 100,000 xg fraction was enriched for exosomal markers and both EV populations immunoblotted for placental proteins, HLA-G and PLAP. However, the vesicles that were isolated by high speed centrifugation were unique in their association with fibronectin. Recently, Jeppesen et al. (2019) described this ECM molecule as a contaminant rather than a component of small EVs isolated from certain cell types. Whether or not this is true for the cytotrophoblast 100,000 xg fraction, which also contained integrins that bind this molecule, remains to be determined. In either case, since we had too little starting material to implement additional purification steps beyond centrifugation, these EVs likely included other associated rather than intrinsic components.

Third decision letter

MS ID#: DEVELOP/2019/187013

MS TITLE: Cytotrophoblast extracellular vesicles enhance decidual cell secretion of immune modulators via TNF-alpha

AUTHORS: Sara K Taylor, Sahar Houshdaran, Joshua F Robinson, Matthew J Gormley, Elaine Y Kwan, Mirhan Kapidzic, Birgit Schilling, Linda C Giudice, and Susan J Fisher
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.