nature portfolio

Peer Review File

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript analyses the alterations in a widely used model for gestational diabetes, the injection of streptozotocin to female mice, few days before pregnancy. Streptozotocin causes the loss of pancreatic beta cells and high levels of glucose in blood during pregnancy that provokes . a well-defined diabetic embryopathy, with defects in yolk sack vasculogenesis. It is also known that in this model, around 25% of the embryos will show neural tube defects (NTD). The authors use a transgenic mouse line to overexpress FGF2 in the endoderm of the yolk sack and they rescue several of the embryonic defects including, neural tube apoptosis and NTD. They also obtain similar results using a transgenic mice overexpressing Survivine, an antiapoptotic factor, in endothelial cells using a Flk-1 promoter. Moreover, they show that FGF2 overexpression in the endoderm restored Survivin expression suppressed by maternal diabetes in Flk-1+ endothelial progenitors. The authors claim that their findings reveal an innovative role for the Survivin-containing exosomes produced by Flk-1+ progenitors in neurulation and indicates that
the endoderm factor FGF2 sustains Survivin expression, a process is compromised by maternal diabetes.
Although the manuscript is interesting and presents a good deal of novelty, the approaches and results do not sustain their conclusions. They have included several approaches and experiments but the impression is that they have not gone fully in detail to understand the developmental alterations of the mice suffering from the high levels of maternal glucose.
The main weaknessess of the manuscript are:
1. The result section is poorly written with very little explanation of the diabetic model they are using; unclear specification of the kind of approaches taken for every figure (in vivo or in vitro) and if the experiment was done in vivo it is not clear if the samples are taken from the embryo, the yolk sack and at which stage of development. In summary, the description of the results is very confusing and makes very difficult to be convinced by their conclusions.
2. The in vivo approaches are supported by the use of two transgenic mice lines, one that express FGF2 under the control of an visceral endoderm enhancer (derived from Transthyretin gene) and other that express Survivine in endothelial cells using a Flk-1 enhancer (not clear if this is exclusive of yolk sack endothelial cells or also drives expression in the embryonic endothelial cells). The manuscript provide very few data on the exact enhancer region used, how many transgenic lines has been used; a proof of which are the cells that express the transgene and a proof of the specificity of the transgene expression. These aspects are essential for the credibility and understanding of their results.
3. The experiments do not solidly proof that there is a communication from the endothelial cells of the yolk sack to the neuroepithelial cells of the embryo throughout exosomes liberated into the yolk sack fluid, that should cross the amnion and be internalized by neuroepitlelial cells. Could it just be that the NTD phenotype they observed in the embryos from diabetic dams is just the consequence of a defect in blood vessel formation in the yolk sack, provoking embryo lack of nutrients supply, lack of hematopoietic cells and consequently a general developmental delay (including delay in neural tube closure, defects in embryonic vasculature formation, etc.) and embryonic death ?.
4. One of their phenotypic output, the neural tube closure defects, is overlooked in a very simplistic way. NTDs can represent many different alterations: cell death in specific neural tube areas, failure in cell adhesion, failure in cytoskeletal proteins and extracellular matrix proteins involved in cell tension and cell movement, etc. They can also represent simply a delay in development. The manuscript should try to address at least some of these aspects.

Other important issues:
1. In Fig. 1A, B, C. They do not explain in which tissue (yolk sack, whole embryo) is FGF2 protein and mRNA levels and DNA methylation altered.
2. In Fig 2 A, the embryo in the picture looks like a 6 to 7 dpc more than a 8 dpc mice. The picture is not convincing of the specificity of the FGF2 transgenic expression. They should provide sections to proof specific expression in visceral endoderm (like in Kwon and Hadjantonakis, Genesis 2009) and

also it is not clear if the transgenic FGF2 mRNA levels are really increased in none diabetic-dams derived embryos (In Fig2 B or 3A, there are not statistically significant differences).
3. In general, it is not clear if the western blot analysis are done using cell lines, yolk sack or embryos. Like those in Fig. S1, A, B, D, F and G.
4. In 2C is difficult to appreciate the recovery of the Flk-1 cells. They should also show a closer view to show if there is a reduction in the number of Flk-1 cells. It is not clear in the graph in Fig2C if the number of cells indicated are per section, per yolk sack or other reference. Similarly, changes in blood islands cannot be seen in Fig2 D. A higher magnification is required.
The pictures shown in Fig2 E and F correspond to embryos of around E10 to E10.5 dpc and not 8.5. In Fig. 2G they should indicate in the graph or in the figure legend what do they mean by blood vessel density. How do they calculate it?
5. In Fig 3C it is not clear what is the anterior posterior level of the sections. Are all sections from non NTD mice or have they chosen the NTD mice?
6. In Fig 5A, they should show co-staining of GFP with Flk-1 inmunostaining. It is not clear in the picture if there is also some transgenic expression in the embryo itself and not only in the yolk sack. In Fig 5F and H is not convincing the rescue of DM mice with Survivine, as the blood vessels in the yolk sack seem very abnormal in F and the forebrain and midbrain development in H is also abnormal compared with even the DM WT.

7. In Fig. 7A: It is not clear if we are looking at exosomes, stained with what marker? In Fig 7C, what is the red staining showing?

8. Fig. S4 is not convincing at all. In S4A they should show the whole neural tube section, and indicate the A-P level. It is not stablished that Nestin could be expressed in such early neuroepithelial cells (8 dpc). Nestin expression appear at around 12.5 dpc. The electron microscopy images should show the whole cell. It is very difficult to determine what are we looking at in those images. In this regard, the CD36 positive exosomes with Survivine could be derived from embryonic cells that express the transgenic Survivine.

9. Regarding the experiments using the GW4869 exosome inhibitor, they only proof that exosomes or multivesicular bodies (that could be generated in many different embryonic and extraembryonic cells) are important for general development, including neural tube closure.

Reviewer #2 (Remarks to the Author):

Manuscript Number: COMMSBIO-20-2945-T

Title: Disruptive exosomal regulation and communication across the three germ layers in embryonic anomalies of diabetic pregnancy

Songying Cao et al. provide rich evidence that exosomes act as carriers for Survivin, an anti-apoptotic protein that is required for normal embryogenesis. The role of Survivin was recognized earlier, however, the involvement of exosomes is a new and highly interesting finding. The study is well designed, the many results are consistent and support the conclusions. Statistical analyses of the results are included and appropriate. Experiments were done in good numbers of replicates. A whole cascade of events in diabetic complications is elaborated including suppression of FGF2 expression by promoter hypermethylation, reduced expression of Survivin leading to neural tube defects. The manuscript is almost ready for publication.

A major question remains, why the rate of embryos developing NTD is relatively low. Only about one

quarter of mice with experimentally induced diabetes mellitus develop NTD. Similarly, usage of exosome inhibitors induced damage only in less than 10% of embryos. Are there alternative pathways to avoid damage, or are the experimental conditions responsible for the low rates? This point should be discussed.

Minor points and mistakes:

It is not clear, what was used as control in the experiments using recombinant FGF2 shown in Figures 4F and 4H.

Line 121: Please check whether 'E5.75' is correct.

Line 281: 'FGF2 exerts both autocrine and porcine functions during embryonic development.' – probably not porcine.

Line 302: '...in the neuroepithelium, which in the innermost layer of the embryo.'

Line 411: 'After washing three times with PBS, samples were incubated with 488 and 594 secondary antibody ...' – not an usual way to specify antibodies.

Line 534: 'Table S1. FGF2 overexpression during vasculogenesis ameliorates maternal diabetes-induced.'

Line 536: 'Table S3. Microinject exosomes to embryos restores NTDs.' Please check grammar.

Figure 4H: Glucose (Mm) should be (mM)

Reviewer #3 (Remarks to the Author):

Defective neurulation in the neuroectoderm leads to neural tube defects (NTD) in diabetic pregnancy. The function of neuroectoderm is tightly regulated by signals from the yolk sac mesoderm and other germ layers. This manuscript convincingly showed that endoderm-derived FGF2 increased the number of Flk1-positive progenitors and vasculogeneis in yolk sac, and promoted transcription of Survivin in Flk1-positive progenitors and increased Survivin levels in the exosomes released from Flk1-positive progenitors. The Survivin-containing exosomes were taken by neuroepithelial cells and the released Survivin promoted neurulation. The FGF2 (endoderm)/Survivin-containing exosomes (mesoderm)/nurulation (neuroectoderm) pathway was inhibited by maternal diabetes, thus leading to NTD. Targeted overexpression of FGF2 or Survivin corrected NTD. The findings conceptually advance our understanding of the molecular mechanisms of neural tube formation, and provide potential novel targets for the treatment of diabetes-induced NTD. The experiments were well designed and carried out and the results are convincing.

Specific comments:

1. Line 107 and Figure 1A and 1B: Since only one time point (E7.5) was examined for FGF2 expression, it may not be appropriate to say "transiently". Therefore, this reviewer suggests deleting "transiently".

2. Please use lines to show the sectioning planes in Figure 3D as they are shown in Figure 6C.

3. Lines 174-177 and Figures 4B-4E: Please mention what cells were used.

4. Figure 4I is supposed to show the numbers of and Surviviin levels in the exosomes released from Flk-1+ progenitors isolated from embryos of diabetic dams with and without FGF2 transgene. It looks like that wrong figures were used for Figure 4I.

5. Fgf2 knockdown efficiency should be shown in Figure 4E.

6. Molecular weights should be indicated in all the WB.

7. Scale bars are presented in most but not all of the images.

Reviewers' comments:

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The manuscript analyses the alterations in a widely used model for gestational diabetes, the injection of streptozotocin to female mice, few days before pregnancy. Streptozotocin causes the loss of pancreatic beta cells and high levels of glucose in blood during pregnancy that provokes a well-defined diabetic embryopathy, with defects in yolk sack vasculogenesis. It is also known that in this model, around 25% of the embryos will show neural tube defects (NTD). The authors use a transgenic mouse line to overexpress FGF2 in the endoderm of the yolk sack and they rescue several of the embryonic defects including, neural tube apoptosis and NTD. They also obtain similar results using a transgenic mice overexpressing Survivin, an antiapoptotic factor, in endothelial cells using a Flk-1 promoter. Moreover, they show that FGF2 overexpression in the endoderm restored Survivin expression suppressed by maternal diabetes in Flk-1+ endothelial progenitors. The authors claim that their findings reveal an innovative role for the Survivin-containing exosomes produced by Flk-1+ progenitors in neurulation and indicates that the endoderm factor FGF2 sustains Survivin expression, a process is compromised by maternal diabetes. Although the manuscript is interesting and presents a good deal of novelty, the approaches and results do not sustain their conclusions. They have included several approaches and experiments but the impression is that they have not gone fully in detail to understand the developmental alterations of the mice suffering from the high levels of maternal glucose.
The main weaknesses of the manuscript are:
1. The result section is poorly written with very little explanation of the diabetic model they are using; unclear specification of the kind of approaches taken for every figure (in vivo or in vitro) and if the experiment was done in vivo it is not clear if the samples are taken from the embryo, the yolk sack and at which stage of development. In summary, the description of the results is very confusing and makes very difficult to be convinced by their conclusions.

Response: We have now included the description of the diabetic embryopathy model in the Methods section. The model has been well established. We clarified whether the experiments are in vivo or in vitro, the tissues are whole embryo or yolk sac and their embryonic days in the text and the figure legends.

2. The in vivo approaches are supported by the use of two transgenic mice lines, one that express FGF2 under the control of an visceral endoderm enhancer (derived from Transthyretin gene) and other that express Survivin in endothelial cells using a Flk-1 enhancer (not clear if this is exclusive of yolk sack endothelial cells or also drives expression in the embryonic endothelial cells). The manuscript provides very few data on the exact enhancer region used, how many transgenic lines has been used; a proof of which are the cells that express the transgene and a proof of the specificity of the transgene expression. These aspects are essential for the credibility and understanding of their results.

Response: We have provided the details of the promoters in the Methods. Numbers of founders generated for the FGF2 transgenic (Tg) line and the Survivin Tg line were provided. Experiments were done by selecting one line for each strain, which can restore FGF2 or survivin expression in embryos exposed to maternal diabetes. We have provided additional data (Figures, S1, S4) to show that visceral endoderm cells expressed the *Fgf2* transgene and the Flk-1 positive progenitors expressed the *Survivin*

transgene at neurulation stage embryos, when embryonic Flk-1 positive progenitors have not yet observed.

3. The experiments do not solidly proof that there is a communication from the endothelial cells of the yolk sac to the neuroepithelial cells of the embryo throughout exosomes liberated into the yolk sack fluid, that should cross the amnion and be internalized by neuroepithelial cells. Could it just be that the NTD phenotype they observed in the embryos from diabetic dams is just the consequence of a defect in blood vessel formation in the yolk sack, provoking embryo lack of nutrients supply, lack of hematopoietic cells and consequently a general developmental delay (including delay in neural tube closure, defects in embryonic vasculature formation, etc.) and embryonic death ?

Response: Using the currently available technologies, we have provided strong evidence that the transgene Survivin was a functional exosomal cargo and presented in the developing neuroepithelium. Injected Survivin-contained exosomes into the amnionic sac prevented NTDs. Electronic microscope examination found exosome-enclosed Survivin protein in neuroepithelial cells. Nevertheless, these evidence cannot completely exclude the positive effect of restored yolk sac vasculature on neurulation. Therefore, we have discussed this limitation in the Discussion (Paragraph 7).

4. One of their phenotypic output, the neural tube closure defects, is overlooked in a very simplistic way. NTDs can represent many different alterations: cell death in specific neural tube areas, failure in cell adhesion, failure in cytoskeletal proteins and extracellular matrix proteins involved in cell tension and cell movement, etc. They can also represent simply a delay in development. The manuscript should try to address at least some of these aspects.

Response: We have addressed one of the underlying mechanisms in NTD formation throughout all experiments: maternal diabetes-induced endoplasmic reticulum stress leads to neuroepithelial cell death detected by the TUNEL assay. We did not observe developmental delays at the neurulation stage.

Other important issues:
1. In Fig. 1A, B, C. They do not explain in which tissue (yolk sack, whole embryo) is FGF2 protein and mRNA levels and DNA methylation altered.

Response: Due to very limited amount tissues of the early stage embryos, all these indices were done in whole embryos.

2. In Fig 2 A, the embryo in the picture looks like a 6 to 7 dpc more than a 8 dpc mice. The picture is not convincing of the specificity of the FGF2 transgenic expression. They should provide sections to proof specific expression in visceral endoderm (like in Kwon and Hadjantonakis, Genesis 2009) and also it is not clear if the transgenic FGF2 mRNA levels are really increased in none diabetic-dams derived embryos (In Fig2 B or 3A, there are not statistically significant differences).

Response: We confirmed that this embryo is at E8.0. Additional experiments as suggested by the review were performed and new data (new Fig. S1) demonstrated visceral endoderm expression of the *Fgf2* transgene. The transgenic line founder was selected based on its ability to restore diabetes-repressed

FGF2 expression. FGF2 Tg had a slight but insignificant increase of FGF2 mRNA (Fig. 2B) but a significant increase of FGF2 protein (Fig. 3A) under nondiabetic conditions.

3. In general, it is not clear if the western blot analysis are done using cell lines, yolk sack or embryos. Like those in Fig. S1, A, B, D, F and G.

Response: We have indicated the tissue or cell source of the experiments in figure legends and in the Results. Fig. S1 is now Fig. S3.

4. In 2C is difficult to appreciate the recovery of the Flk-1 cells. They should also show a closer view to show if there is a reduction in the number of Flk-1 cells. It is not clear in the graph in Fig2C if the number of cells indicated are per section, per yolk sack or other reference. Similarly, changes in blood islands cannot be seen in Fig2 D. A higher magnification is required.
The pictures shown in Fig2 E and F correspond to embryos of around E10 to E10.5 dpc and not 8.5. In Fig. 2G they should indicate in the graph or in the figure legend what do they mean by blood vessel density. How do they calculate it?

Response: We have added details of the Flk-1 cell number, the blood island number and blood density measurement in the Figure 2 legend. Blood islands were pointed by arrows in Fig. 2D. The stage of the embryos is corrected.

5. In Fig 3C it is not clear what is the anterior posterior level of the sections. Are all sections from non NTD mice or have they chosen the NTD mice?

Response: Experiments were done in E8.5 before neural tube closure and thus we do not know which one become NTD embryo. However, published data from others and us have demonstrated that neuroepithelial cell apoptosis is observed in all embryos exposed to diabetes. Only a portion of embryos whose neuroepithelial cell apoptosis reaches a threshold exhibits NTDs. Our previously studies (Nat Commun. 2019 Jan 17;10(1):282; Nat Commun. 2017 May 5;8:15182.) have demonstrated that the section at the midbrain-hindbrain boundary exhibits the highest number of apoptotic cells. The embryo was sectioned coronally at the midbrain-hindbrain boundary.

6. In Fig 5A, they should show co-staining of GFP with Flk-1 immunostaining. It is not clear in the picture if there is also some transgenic expression in the embryo itself and not only in the yolk sac. In Fig 5F and H is not convincing the rescue of DM mice with Survivin, as the blood vessels in the yolk sack seem very abnormal in F and the forebrain and midbrain development in H is also abnormal compared with even the DM WT.
Response: In Fig. 5A, it showed GFP staining not Flk-1 staining. GFP is co-expressed with the transgene c-myc-tagged survivin. We have provided additional data in new Fig. S2 to show survivin transgene expression in the yolk sac Flk-1 positive cells. At E8.0, we did not observe any Flk1 positive cells in the embryo. In Fig. 5G of the DM plus survivin group, large vessel in the yolk sac is clearly visible whereas in the DM group, the yolk sac does not have any vessel. It is the same in Fig. 5G.

7. In Fig. 7A: It is not clear if we are looking at exosomes, stained with what marker? In Fig 7C, what is the red staining showing?
Response: In Fig. 7A, Exosomes (bright round dots) were directly detected by the NanoSight (#NS300, Malvern Panalytical). The neural stem cells are labeled by nestin immunostaining in Red. These information were added into the Figure legend.

8. Fig. S4 is not convincing at all. In S4A they should show the whole neural tube section, and indicate the A-P level. It is not stablished that Nestin could be expressed in such early neuroepithelial cells (8 dpc). Nestin expression appear at around 12.5 dpc. The electron microscopy images should show the whole cell. It is very difficult to determine what are we looking at in those images. In this regard, the CD36 positive exosomes with Survivin could be derived from embryonic cells that express the transgenic Survivin.
Response: It has been demonstrated that nestin is expressed from E7.75 in the mouse embryo (Brain Res Dev Brain Res. 1995 Jan 14;84(1):109-29.).

The rat nestin promoter we received from Dr. Weimin Zhong (Nature. 2002 Oct 31;419(6910):929-34.) at Yale University drives transgene expression from E8.0 onwards, which is also proved by our previous studies (*Nat Commun*. 2017; 8: 15182.).

Again, in E8.0, we did not observe any Flk1 positive cells in the embryo and thus the survivin protein detected in the neuroepithelial cells surrounding by the exosome marker CD36 is most likely from the yolk sac Flk1 positive cells. Nevertheless, we toned down our conclusion.

9. Regarding the experiments using the GW4869 exosome inhibitor, they only proof that exosomes or multivesicular bodies (that could be generated in many different embryonic and extraembryonic cells) are important for general development, including neural tube closure.

Response: Yes, we agree and have toned down our conclusion.

Reviewer #2 (Remarks to the Author):

Manuscript Number: COMMSBIO-20-2945-T

Title: Disruptive exosomal regulation and communication across the three germ layers in embryonic anomalies of diabetic pregnancy

Songying Cao et al. provide rich evidence that exosomes act as carriers for Survivin, an anti-apoptotic protein that is required for normal embryogenesis. The role of Survivin was recognized earlier, however, the involvement of exosomes is a new and highly interesting finding. The study is well designed, the many results are consistent and support the conclusions. Statistical analyses of the results are included and appropriate. Experiments were done in good numbers of replicates. A whole cascade of events in

diabetic complications is elaborated including suppression of FGF2 expression by promoter hypermethylation, reduced expression of Survivin leading to neural tube defects. The manuscript is almost ready for publication.

A major question remains, why the rate of embryos developing NTD is relatively low. Only about one quarter of mice with experimentally induced diabetes mellitus develop NTD. Similarly, usage of exosome inhibitors induced damage only in less than 10% of embryos. Are there alternative pathways to avoid damage, or are the experimental conditions responsible for the low rates? This point should be discussed.

Response: We have discussed these points (please see paragraph 7 in the Discussion)

Minor points and mistakes:

It is not clear, what was used as control in the experiments using recombinant FGF2 shown in Figures 4F and 4H.
Response: We have corrected to vehicle (indicated in the figure legend as water)
Line 121: Please check whether 'E5.75' is correct.
Response: Yes, it is correct.
Line 281: 'FGF2 exerts both autocrine and porcine functions during embryonic development.' – probably not porcine.
Response: We have corrected to "paracrine".
Line 302: '...in the neuroepithelium, which in the innermost layer of the embryo.'
Response: corrected to "which is".
Line 411: 'After washing three times with PBS, samples were incubated with 488 and 594 secondary antibody ...' – not an usual way to specify antibodies.
Response: Changed to "Alexa Fluor 488 or 594-conjugated".
Line 534: 'Table S1. FGF2 overexpression during vasculogenesis ameliorates maternal diabetes- induced.'
Response: Changed to "maternal diabetes-induced NTDs".
Line 536: 'Table S3. Microinject exosomes to embryos restores NTDs.' Please check grammar.
Response: corrected.
Figure 4H: Glucose (Mm) should be (mM)
Response: corrected.

Reviewer #3 (Remarks to the Author):

Defective neurulation in the neuroectoderm leads to neural tube defects (NTD) in diabetic pregnancy. The function of neuroectoderm is tightly regulated by signals from the yolk sac mesoderm and other germ layers. This manuscript convincingly showed that endoderm-derived FGF2 increased the number of Flk1-positive progenitors and vasculogenesis in yolk sac, and promoted transcription of Survivin in Flk1-positive progenitors and increased Survivin levels in the exosomes released from Flk1-positive progenitors. The Survivin-containing exosomes were taken by neuroepithelial cells and the released

Survivin promoted neurulation. The FGF2 (endoderm)/Survivin-containing exosomes (mesoderm)/neurulation (neuroectoderm) pathway was inhibited by maternal diabetes, thus leading to NTD. Targeted overexpression of FGF2 or Survivin corrected NTD. The findings conceptually advance our understanding of the molecular mechanisms of neural tube formation, and provide potential novel targets for the treatment of diabetes-induced NTD. The experiments were well designed and carried out and the results are convincing.

Specific comments:

1. Line 107 and Figure 1A and 1B: Since only one time point (E7.5) was examined for FGF2 expression, it may not be appropriate to say "transiently". Therefore, this reviewer suggests deleting "transiently".
Response: deleted.
2. Please use lines to show the sectioning planes in Figure 3D as they are shown in Figure 6C.
Response: added.
3. Lines 174-177 and Figures 4B-4E: Please mention what cells were used.
Response: mentioned.
4. Figure 4I is supposed to show the numbers of and Survivin levels in the exosomes released from Flk-1+ progenitors isolated from embryos of diabetic dams with and without FGF2 transgene. It looks like that wrong figures were used for Figure 4I.
Response: We have corrected as following: Exosome number and Survivin protein expression in exosomes of isolated E8 Flk-1^{+} progenitors treated by Re-FGF2 (H) or si-FGF2 (I).
5. Fgf2 knockdown efficiency should be shown in Figure 4E.
Response: mentioned in the figure legend.
6. Molecular weights should be indicated in all the WB.
Response: indicated in the last supplementary figure.
7. Scale bars are presented in most but not all of the images.
Response: added when it is possible.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In my view, all the main concerns previously raised, has been satisfactorily addressed in the new improved version of the manuscript.
Minor comment: Please correct grammar and spelling in the new paragraph in the Discussion section: ..."into the amnionic sac reduces NTD incidences..."

Reviewer #2 (Remarks to the Author):

Manuscript # COMMSBIO-20-2945A

Title: Functional cargos of exosomes derived from Flk-1+ vascular 1 progenitors enable neurulation and ameliorate embryonic anomalies in diabetic pregnancy

In their revised version of the manuscript, Songying Cao et al. did some small corrections, however, some major issues were not addressed at all.
Differently than stated in their rebuttal, the question why the rate of embryos developing NTD is relatively low in their model is not discussed.
Furthermore, Figures 4F and 4H remained unchanged and still contain a control (in ng/ml or 10ng/ml), which was water (vehicle control). Also the legend was not improved.

Response to the reviewers' comments

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In my view, all the main concerns previously raised, has been satisfactorily addressed in the new improved version of the manuscript.
Minor comment: Please correct grammar and spelling in the new paragraph in the Discussion section:
..."into the amnionic sac reduces NTD incidences..."

Response: corrections have been made.

Reviewer #2 (Remarks to the Author):

Manuscript # COMMSBIO-20-2945A

Title: Functional cargos of exosomes derived from Flk-1+ vascular 1 progenitors enable neurulation and ameliorate embryonic anomalies in diabetic pregnancy

In their revised version of the manuscript, Songying Cao et al. did some small corrections, however, some major issues were not addressed at all.

Response: we respectfully disagree with the reviewer. We have made extensive changes in the first revision and added additional data (supplementary figure 1 and 4).

Differently than stated in their rebuttal, the question why the rate of embryos developing NTD is relatively low in their model is not discussed.

A major question remains, why the rate of embryos developing NTD is relatively low. Only about one quarter of mice with experimentally induced diabetes mellitus develop NTD. Similarly, usage of exosome inhibitors induced damage only in less than 10% of embryos. Are there alternative pathways to avoid damage, or are the experimental conditions responsible for the low rates? This point should be discussed.

Response: We have discussed these points (please see paragraph 8 in the Discussion).

The NTD rates in this study are in line with published studies from us and others. In human, maternal diabetes does not induce 100% of birth defects. Maternal diabetes-induced cellular stress levels need reach a threshold that leads to the formation of a birth defect. Clinical studies

have demonstrated that hyperglycemia levels of maternal diabetes are positively associated with the incidence of birth defects. In an in vitro animal embryo culture study demonstrate that at the level of 750 mg/dl glucose, all embryos are defective. Such level of hyperglycemia is not realistic in human conditions. The hyperglycemia level of between 350-450 mg/dl in our mouse model of diabetic embryopathy is in the range of the hyperglycemia levels in pregnant women with pregestational maternal diabetes. Thus, our model faithfully reflects the human conditions. Likewise, the dose of the exosome inhibitor is at a low level to avoid any adverse maternal impact. Higher level of the exosome inhibitor could lead to higher NTD incidence. Nevertheless, the exosome inhibitor study shows that exosome production is critical for neurulation.

Furthermore, Figures 4F and 4H remained unchanged and still contain a control (in ng/ml or 10ng/ml), which was water (vehicle control). Also the legend was not improved.
Response: Sorry for not correcting in the first revision. Corrections are made.