THE ROYAL SOCIETY PUBLISHING

PROCEEDINGS B

The active role of spermatozoa in transgenerational inheritance

Ilaria Sciamanna, Annalucia Serafino, James A. Shapiro and Corrado Spadafora

Article citation details
Proc. R. Soc. B **286**: 20191263.
http://dx.doi.org/10.1098/rspb.2019.1263

Review timeline
Original submission: 29 May 2019
1st revised submission: 17 July 2019
2nd revised submission: 6 August 2019
Final acceptance: 6 August 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2019-1263.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Marginal

General interest: Is the paper of sufficient general interest?
Acceptable

Quality of the paper: Is the overall quality of the paper suitable?
Marginal

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
Sciamanna et al. reviews some of the literature on sperm as a carrier of transgenerational information. Overall the manuscript is well written, however, it lacks a concise message weaving in-and-out of soma-to-germline communication of extracellular vesicles and RNAs, to uptake of nucleic acids by sperm, which as presented do not seem totally related. As noted by the authors the first is a naturally occurring mechanism of communication while the latter is a phenomenon only present in the absence of seminal fluid (a totally non-natural and manufactured situation). Additionally, the citations of this review are a mess for two reasons -with some examples below (several more could be pointed out redundantly). The first simply being that there are many situations where citations are left out or in the wrong place. The second being that there are several instances where the authors cite biology that occurs in somatic cell types (not sperm) and apply those findings to sperm biology without making it clear that said biology has not yet been demonstrated in the germline or sperm.

Page 3, line 49-51. 17 and 23 should be also be cited here, as well as PMID: 29472946 from the Homanics lab.

Page 3, line 63. As presented, Citation 6 does not fit here. The way it is presented it infers this citation demonstrates soma-to-sperm delivery of RNA by extracellular vesicles, which it does not.

Page 3, line 64-66. Citations 18-21 do not demonstrate the statement they are associated with.

Page 4, lines 78-80. This is another example of a statement that is not supported by its citations. Citations 33 & 34 having nothing to do with stress or 'donor health' affecting the composition of RNAs in extracellular vesicles delivered to sperm.

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This is a succinct review of research focusing on the uptake and functional implications of nucleic acids by mammalian spermatozoa. Although this topic has been the subject of numerous recent reviews, particularly in the context of extracellular vesicle-mediated delivery mechanisms, there are several points of novelty that set this article apart from previous reviews. These include the consideration given to the ability of spermatozoa to directly incorporate exogenous DNA independent of extracellular vesicles and the fate of this DNA upon delivery to the oocyte. Listed below are specific comments in which I encourage the authors to consider tempering some of their claims for phenomena that have yet to be substantiated by definitive experimental evidence.

Specific comments
1. L49: Please reword the sentence "…the regulatory RNA content is specifically mediated by the epididymosomes…" to "…the regulatory RNA content is selectively modified by interaction with

epididymosomes…" . As it is currently worded, this statement appears to overlook the importance of the abundant endogenous RNA that testicular sperm harbor prior to entering the epididymis and encountering epididymosomes.

2. L78: replace 'regular' with 'regulating'

3. L79-86: I have reservations as to whether sufficient evidence exists to support the claim that "extracellular vesicles can deliver many different RNAs to sperm from 'diverse cellular sources'…." Noting that the two cited references describe vesicles derived from endothelial (33) and glioblastoma (34) cells, and that neither reported transfer of RNA cargo to sperm cells, I think this information is potentially misleading. Although this mode of information transfer remains a distinct possibility and warrants mention as an intriguing area for future investigation, to the best of my knowledge the single study cited in reference [35] is currently the only experimental evidence to suggest that sperm can act as recipients for extracellular vesicles originating from beyond the epididymis. The interpretation of these data is also somewhat confounded by the potential for the inoculated melanoma cells to have metastasized or themselves been delivered directly to the epididymis. I would therefore encourage the authors to either temper, or provide additional evidence to substantiate, their claim that "RNA-based information can arrive at epididymal spermatozoa even when released from a distant tissue".

4. L117-118: The data presented in Fig. 1d-f are compelling, but there remains some contention as to whether exosomes are capable of being directly "…taken up and internalized in sperm heads". Since sperm lack endocytotic machinery, it has instead been reasoned that exosomes may transiently fuse with the sperm membrane to deliver their cargo before being released. In the absence of detailed description of the methodology used, it is not clear what form of rhodamine was used and whether this remained unbound or bound DNA/RNA/protein within the exosomes. In any case, on the available evidence one cannot conclude that the entire exosome was 'taken up and internalized' so this sentence may need to be reworded to "Similarly to DNA, foreign exosomes can interact and deliver their cargo to sperm heads".

Decision letter (RSPB-2019-1263.R0)

04-Jul-2019

Dear Prof Spadafora:

Your manuscript has now been peer reviewed and I have assessed the reports. The reviewers' comments (not including confidential comments to the Editor) are included at the end of this email for your reference. As you will see, the reviewers have raised some concerns with your manuscript and, currently, I cannot accept it for publication -- the main issue is a lack of clarity in places about whether there is direct or only circumstantial evidence for various claims, and thus a distinction between what is speculation, yet to be tested, and what is a review of solid evidence. However, it is possible that the manuscript could be revised to remedy this, so I would be happy to consider a resubmission.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Innes Cuthill

Prof. Innes Cuthill,
Reviews Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
Sciamanna et al. reviews some of the literature on sperm as a carrier of transgenerational information. Overall the manuscript is well written, however, it lacks a concise message weaving in-and-out of soma-to-germline communication of extracellular vesicles and RNAs, to uptake of nucleic acids by sperm, which as presented do not seem totally related. As noted by the authors the first is a naturally occurring mechanism of communication while the latter is a phenomenon only present in the absence of seminal fluid (a totally non-natural and manufactured situation). Additionally, the citations of this review are a mess for two reasons -with some examples below (several more could be pointed out redundantly). The first simply being that there are many situations where citations are left out or in the wrong place. The second being that there are several instances where the authors cite biology that occurs in somatic cell types (not sperm) and apply those findings to sperm biology without making it clear that said biology has not yet been demonstrated in the germline or sperm.

Page 3, line 49-51. 17 and 23 should be also be cited here, as well as PMID: 29472946 from the Homanics lab.

Page 3, line 63. As presented, Citation 6 does not fit here. The way it is presented it infers this citation demonstrates soma-to-sperm delivery of RNA by extracellular vesicles, which it does not.

Page 3, line 64-66. Citations 18-21 do not demonstrate the statement they are associated with.

Page 4, lines 78-80. This is another example of a statement that is not supported by its citations. Citations 33 & 34 having nothing to do with stress or 'donor health' affecting the composition of RNAs in extracellular vesicles delivered to sperm.

Referee: 2

Comments to the Author(s)
This is a succinct review of research focusing on the uptake and functional implications of nucleic acids by mammalian spermatozoa. Although this topic has been the subject of numerous recent reviews, particularly in the context of extracellular vesicle-mediated delivery mechanisms, there are several points of novelty that set this article apart from previous reviews. These include the consideration given to the ability of spermatozoa to directly incorporate exogenous DNA independent of extracellular vesicles and the fate of this DNA upon delivery to the oocyte. Listed below are specific comments in which I encourage the authors to consider tempering some of their claims for phenomena that have yet to be substantiated by definitive experimental evidence.

Specific comments
1. L49: Please reword the sentence "…the regulatory RNA content is specifically mediated by the epididymosomes…" to "…the regulatory RNA content is selectively modified by interaction with epididymosomes…" . As it is currently worded, this statement appears to overlook the importance of the abundant endogenous RNA that testicular sperm harbor prior to entering the epididymis and encountering epididymosomes.

2. L78: replace 'regular' with 'regulating'

3. L79-86: I have reservations as to whether sufficient evidence exists to support the claim that "extracellular vesicles can deliver many different RNAs to sperm from 'diverse cellular sources'…." Noting that the two cited references describe vesicles derived from endothelial (33) and glioblastoma (34) cells, and that neither reported transfer of RNA cargo to sperm cells, I think this information is potentially misleading. Although this mode of information transfer remains a distinct possibility and warrants mention as an intriguing area for future investigation, to the best of my knowledge the single study cited in reference [35] is currently the only experimental evidence to suggest that sperm can act as recipients for extracellular vesicles originating from beyond the epididymis. The interpretation of these data is also somewhat confounded by the potential for the inoculated melanoma cells to have metastasized or themselves been delivered directly to the epididymis. I would therefore encourage the authors to either temper, or provide additional evidence to substantiate, their claim that "RNA-based information can arrive at epididymal spermatozoa even when released from a distant tissue".

4. L117-118: The data presented in Fig. 1d-f are compelling, but there remains some contention as to whether exosomes are capable of being directly "…taken up and internalized in sperm heads". Since sperm lack endocytotic machinery, it has instead been reasoned that exosomes may transiently fuse with the sperm membrane to deliver their cargo before being released. In the absence of detailed description of the methodology used, it is not clear what form of rhodamine was used and whether this remained unbound or bound DNA/RNA/protein within the exosomes. In any case, on the available evidence one cannot conclude that the entire exosome was 'taken up and internalized' so this sentence may need to be reworded to "Similarly to DNA, foreign exosomes can interact and deliver their cargo to sperm heads".

Author's Response to Decision Letter for (RSPB-2019-1263.R0)

See Appendix A.

RSPB-2019-1263.R1 (Revision)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Marginal

General interest: Is the paper of sufficient general interest?
Acceptable

Quality of the paper: Is the overall quality of the paper suitable?
Marginal

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
My comments regarding citations have been adequately addressed. I have one remaining issue – throughout the manuscript the authors use the term 'microvesicle' as a blanket term for an extracellular vesicle (i.e. exosomes or microvesicle). This is incorrect usage of the fields nomenclature, I believe the blanket term should be 'extracellular vesicle', where a microvesicle is distinct term used for a vesicle of plasma membrane origin and exosome of endosomal origin. This discrepancy is particularly evident in the second paragraph of the introduction, but also occurs on a few other occasions in the text.

Decision letter (RSPB-2019-1263.R1)

06-Aug-2019

Dear Dr Spadafora

I am pleased to inform you that your manuscript RSPB-2019-1263.R1 entitled "The active role of spermatozoa in transgenerational inheritance" has been accepted for publication in Proceedings B.

We used just one referee, and he/she has recommended publication but also requests a change in terminology from microvesicle to 'extracellular vesicle'. Therefore, I invite you to respond to the referee's comment and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into https://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Before uploading your revised files please make sure that you have:

1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".

2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. All supplementary materials

accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

5) Data accessibility section and data citation
It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository.

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
• DNA sequences: Genbank accessions F234391-F234402
• Phylogenetic data: TreeBASE accession number S9123
• Final DNA sequence assembly uploaded as online supplemental material
• Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311
NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available) which will take you to your unique entry in the Dryad repository. If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

6) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit https://royalsociety.org/journals/authors/author-guidelines/.

Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,

Innes

Proc. Innes Cuthill
Reviews Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
My comments regarding citations have been adequately addressed. I have one remaining issue – throughout the manuscript the authors use the term 'microvesicle' as a blanket term for an extracellular vesicle (i.e. exosomes or microvesicle). This is incorrect usage of the fields nomenclature, I believe the blanket term should be 'extracellular vesicle', where a microvesicle is distinct term used for a vesicle of plasma membrane origin and exosome of endosomal origin. This discrepancy is particularly evident in the second paragraph of the introduction, but also occurs on a few other occasions in the text.

Author's Response to Decision Letter for (RSPB-2019-1263.R1)

See Appendix B.

Decision letter (RSPB-2019-1263.R2)

06-Aug-2019

Dear Dr Spadafora

I am pleased to inform you that your manuscript entitled "The active role of spermatozoa in transgenerational inheritance" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact during this period, let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

Your article has been estimated as being 8 pages long. Our Production Office will be able to confirm the exact length at proof stage.

Open access
You are invited to opt for open access via our author pays publishing model. Payment of open access fees will enable your article to be made freely available via the Royal Society website as soon as it is ready for publication. For more information about open access publishing please visit our website at http://royalsocietypublishing.org/site/authors/open_access.xhtml.

The open access fee is £1,700 per article (plus VAT for authors within the EU). If you wish to opt for open access then please let us know as soon as possible.

Paper charges
An e-mail request for payment of any related charges will be sent out shortly. The preferred payment method is by credit card; however, other payment options are available.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,

Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Comment: Sciamanna et al. reviews some of the literature on sperm as a carrier of transgenerational information. Overall the manuscript is well written, however, it lacks a concise message weaving in-and-out of soma-to-germline communication of extracellular vesicles and RNAs, to uptake of nucleic acids by sperm, which as presented do not seem totally related.
As noted by the authors the first is a naturally occurring mechanism of communication while the latter is a phenomenon only present in the absence of seminal fluid (a totally non-natural and manufactured situation).

***Reply**: The reviewer's remark suggests that our text was probably not fully clear. Concerning the binding and internalization of vesicles and nucleic acids in sperm heads: the seminal fluid, present when ejaculated semen is used in fertilization assays, provides a crucial protection barrier. The seminal fluid is instead absent in most in vivo experiments showing epididymis-to-sperm communication. The seminal fluid strongly antagonizes the sperm permeability: no DNA or vesicles are taken up when even trace amounts of seminal fluid are present in the sperm preparation. Specific protein factors ensure the loss of permeability of spermatozoa in the ejaculated semen, and thus preserve the identity and transmission of the paternal genome at the time of fertilisation. The seminal fluid, therefore, must be thoroughly removed in order to restore the permeability in in vitro assays. To that extent, it is true that the restored permeability of mature ejaculated spermatozoa is a biotechnological operation. In contrast, epididymal spermatozoa, e.g. murine spermatozoa surgically withdrawn from epididymis (such as those shown in Fig.1), are spontaneously permeable to molecules and vesicles, both in in vivo (in the epididymis) and in in vitro assays. Thus, the difference in permeability entirely depends on the source of the sperm cells, whether epididymal or ejaculated, with or without seminal fluid.*
The text has been revised and we hope to have now clarified these points (lines 115-127).

Additionally, the citations of this review are a mess for two reasons -with some examples below (several more could be pointed out redundantly). The first simply being that there are many situations where citations are left out or in the wrong place. The second being that there are several instances where the authors cite biology that occurs in somatic cell types (not sperm) and apply those findings to sperm biology without making it clear that said biology has not yet been demonstrated in the germline or sperm.

Comment: Page 3, line 49-51. 17 and 23 should be also be cited here, as well as PMID: 29472946 from the Homanics lab.
Reply: *Ref. 17 and 23 (now 12 and 14 in the revised text) are now properly cited and the article by Romala et al. 2018 (13) included in the list of references as recommended by the reviewer (lines 231-233)*

Comment: Page 3, line 63. As presented, Citation 6 does not fit here. The way it is presented it infers this citation demonstrates soma-to-sperm delivery of RNA by extracellular vesicles, which it does not.
Reply: *We thank the reviewer for that remark. Citation 6 has been removed from that*

particular location in the revised text

Comment: Page 3, line 64-66. Citations 18-21 do not demonstrate the statement they are associated with.
Reply: *the reviewer points out that citations 18-21 do not demonstrate our statement that "a significant proportion of RNA loaded in sperm heads is of somatic origin…..". In strictly literal terms, the reviewer is right. However, we are aware that these papers report evidence of vesicle-mediated and, in our view, it is not unreasonable to speculate from these data that a proportion of sperm-stored RNA is not of germline origin but somatically imported. On these grounds, we took the liberty to interpret the meaning of the data, which in essence suggest that the delivery of the extracellular vesicle RNA-containing cargo can significantly affect the nature of the RNA stored in spermatozoa. Moreover, papers quoted in 18-21 recall that the data, overall, are consistent with the model of Darwinian Pangenesis, a conclusion in further agreement with our interpretation. Having explained our grounds, however, we understand that our original phrasing raised controversy and have therefore removed that statement from the main text.*

Comment: Page 4, lines 78-80. This is another example of a statement that is not supported by its citations. Citations 33 & 34 having nothing to do with stress or 'donor health' affecting the composition of RNAs in extracellular vesicles delivered to sperm.

Reply: *Ref. 33 (now 34) shows that cellular stress can alter the content of exosomes and ref. 34 (now 35) that glioma-specific mRNAs and miRNAs are detected in circulating vesicles, suggesting that the vesicle content is dependent on stressing stimuli and donor health. In the context of this manuscript, we have used these examples in support of the view that exosomes can carry stress-originated and pathological information throughout the blood stream. Building on a growing body of evidence and our own work, we hypothesize that exosomes carrying such "altered" information can reach the epididymis, enter spermatozoa and pass to the next generation. Because these steps were not explicitly mentioned in the original papers, we accept the reviewer's criticism and have reformulated the statement in the revised manuscript (lines 76-84).*

Referee: 2

Comments to the Author(s)
This is a succinct review of research focusing on the uptake and functional implications of nucleic acids by mammalian spermatozoa. Although this topic has been the subject of numerous recent reviews, particularly in the context of extracellular vesicle-mediated delivery mechanisms, there are several points of novelty that set this article apart from previous reviews. These include the consideration given to the ability of spermatozoa to directly incorporate exogenous DNA independent of extracellular vesicles and the fate of this DNA upon delivery to the oocyte. Listed below are specific comments in which I encourage the authors to consider tempering some of their claims for phenomena that have yet to be substantiated by definitive experimental evidence.

Specific comments
Comment: 1. L49: Please reword the sentence "…the regulatory RNA content is specifically mediated by the epididymosomes…" to "…the regulatory RNA content is selectively modified by interaction with epididymosomes…" . As it is currently worded, this statement appears to overlook the importance of the abundant endogenous RNA that

testicular sperm harbor prior to entering the epididymis and encountering epididymosomes.
Reply: *The sentence is now rephrased according to the reviewer's suggestion in the revised manuscript (lines 47-48)*

Comment: 2. L78: replace 'regular' with 'regulating'
Reply: *The sentence starting at line 78 (now 76) has been totally rephrased*

Comment: 3. L79-86: I have reservations as to whether sufficient evidence exists to support the claim that "extracellular vesicles can deliver many different RNAs to sperm from 'diverse cellular sources'…." Noting that the two cited references describe vesicles derived from endothelial (33) and glioblastoma (34) cells, and that neither reported transfer of RNA cargo to sperm cells, I think this information is potentially misleading. Although this mode of information transfer remains a distinct possibility and warrants mention as an intriguing area for future investigation, to the best of my knowledge the single study cited in reference [35] is currently the only experimental evidence to suggest that sperm can act as recipients for extracellular vesicles originating from beyond the epididymis. The interpretation of these data is also somewhat confounded by the potential for the inoculated melanoma cells to have metastasized or themselves been delivered directly to the epididymis. I would therefore encourage the authors to either temper, or provide additional evidence to substantiate, their claim that "RNA-based information can arrive at epididymal spermatozoa even when released from a distant tissue".
Reply: *Similar criticisms on our overinterpretation of citations 33 & 34 (now 34 & 35) were raised by reviewer 1. We have accepted this criticism and have revised the text accordingly. Ref. 33 shows that exosome content is altered in response to cellular stress and ref. 34 that glioma-specific mRNAs and miRNAs are detected in circulating vesicles, suggesting that the vesicle content is dependent on stressing stimuli and donor health. These results clearly showed that stress- and disease-induced RNA-based information can spread throughout the blood stream. A growing body of evidence and our own work prompted us to speculate that exosomes carrying such "altered" information could reach the epididymis, enter spermatozoa and pass to the next generation. Having said that, however, we understand that our original phrasing raised controversy and have therefore removed that statement from the main text.*

Comment: 4. L117-118: The data presented in Fig. 1d-f are compelling, but there remains some contention as to whether exosomes are capable of being directly "…taken up and internalized in sperm heads". Since sperm lack endocytotic machinery, it has instead been reasoned that exosomes may transiently fuse with the sperm membrane to deliver their cargo before being released. In the absence of detailed description of the methodology used, it is not clear what form of rhodamine was used and whether this remained unbound or bound DNA/RNA/protein within the exosomes. In any case, on the available evidence one cannot conclude that the entire exosome was 'taken up and internalized' so this sentence may need to be reworded to "Similarly to DNA, foreign exosomes can interact and deliver their cargo to sperm heads".
Reply: *To the best of our knowledge, no study thus far has systematically characterized the process of internalization of exosomes in sperm heads. It is well known, however, that mature spermatozoa are highly permeable cells, able to internalize and accommodate in their heads not only naked nucleic acid molecules, but also full viral particles (among others see Baccetti et al., HIV-particles in spermatozoa of patients with AIDS and their transfer to oocytes. 1994. J Cell Biol. 127, 903-914). On these grounds, the internalization*

of entire nanovesicles is not an unreasonable possibility. We have now reworded the text as suggested by the reviewer (lines 128-129).

Appendix B

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Comment: My comments regarding citations have been adequately addressed. I have one remaining issue – throughout the manuscript the authors use the term ‘microvesicle’ as a blanket term for an extracellular vesicle (i.e. exosomes or microvesicle). This is incorrect usage of the fields nomenclature, I believe the blanket term should be ‘extracellular vesicle’, where a microvesicle is distinct term used for a vesicle of plasma membrane origin and exosome of endosomal origin. This discrepancy is particularly evident in the second paragraph of the introduction, but also occurs on a few other occasions in the text

***Reply**: Following the reviewer’s suggestion we have amended the manuscript by replacing the term “microvesicles” with “extrachromosomal vesicles” throughout the text. We thank the reviewer for his/her thorough reviewing of the manuscript.*