

The Chromosome 19 miRNA Cluster Guards Trophoblasts Against Overacting Innate Immunity

Corresponding Author: Dr Yoel Sadovsky

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Summary

In this study, Mouillet et al. explored the role of the chromosome 19 miRNA cluster (C19MC), a primate-specific placental microRNA cluster, in modulating innate immune responses. The authors first mapped trophoblast-specific accessible chromatin regions on chromosome 19 and proposed that these regions encompass enhancers regulating C19MC expression. They then performed CRISPR/Cas9-mediated deletions of selected elements in the trophoblast cell line BeWo to assess their functional relevance. Notably, BeWo cells lacking the ATAC-11 region (BeWo-dA11.21 and -dA11.23) exhibited resistance to viral infection and demonstrated spontaneous upregulation of interferons and interferon-stimulated genes (ISGs), which was further enhanced by exposure to synthetic double-stranded RNA, poly(I:C). Using diverse pathogen-associated molecular patterns and analyses of dsRNA localization, the authors provided evidence implicating Toll-like receptor 3 (TLR3) in this amplified innate immune response. They conclude that C19MC protects trophoblasts against excessive innate immune activation during viral infection. The findings are novel and relevant, significantly expanding our understanding of trophoblast biology.

I have several comments, outlined below.

Major Comments

- Mechanistic Evidence and Role of C19MC miRNAs:

While the experiments presented here clearly demonstrate that deletion of an enhancer region for C19MC in trophoblast cells leads to spontaneous activation of interferon-associated pathways and heightened responses to dsRNA, the mechanistic involvement of C19MC miRNAs themselves in regulating these immune responses remains insufficiently substantiated. Much of the proposed mechanism appears to be inferred from prior studies rather than conclusively established in this work. Given the mechanistic emphasis stated in the title of the manuscript, it would considerably strengthen the study if the authors could include experimental evidence directly supporting the role of C19MC miRNAs in these regulatory processes.

- Specificity of dsRNA Receptor Involvement:

The data indicating TLR3 involvement in the observed immune activation are compelling; however, the interpretation warrants some caution. The various dsRNA receptor ligands employed in these experiments are not entirely specific for individual dsRNA sensors and exhibit overlapping recognition profiles. Therefore, concluding that the responses are exclusively mediated by TLR3 may be an overstatement based solely on ligand stimulation. Additionally, although the co-localization of dsRNA with endosomes provides supportive information, it remains indirect and does not constitute definitive evidence of TLR3 engagement. The authors should consider moderating this interpretation or conducting additional experiments, such as knockdown of other dsRNA receptors like RIG-I or MDA5, to clarify receptor specificity.

- Page 5, Line 10: The transition between the discussion of BAC plasmid sequences and the analysis of C19MC expression in primary trophoblasts is somewhat abrupt. Additional context would help improve the coherence and clarity of this section.

- Page 5, Line 18: The rationale for focusing specifically on the autonomous enhancer activity of C19MC regions should be elaborated in more detail.

- Page 6, Lines 9–15: What evidence supports the assumption that ATAC peaks identified in human trophoblasts would demonstrate comparable activity in the mouse placenta? This requires clarification, particularly since C19MC is absent from the mouse genome.

- Page 8, Lines 6–7: It is commendable that BeWo-dA3/4-21 and -41 were included as negative controls to exclude potential off-target effects arising from lentiviral infection. However, did the authors verify the successful deletion of the A3/4 regions in

these two BeWo clones?

- Page 14, Lines 18–20: The logical basis of this sentence is not fully articulated and should be further elaborated to clarify the argument.

Minor Comments

- Page 5, Line 5: Please spell out “BAC” at its first mention.
- Page 5, Line 14: The word “cell” is unnecessary here, as HUVEC already stands for “Human Umbilical Vein Endothelial Cell.”
- Page 15, Line 2: The phrase “[add competition experiment]” appears to have been inadvertently retained and should be removed.
- Nomenclature Consistency: The manuscript inconsistently refers to ATAC-11 (e.g., as “dd11” on Page 13, Line 25, and as “A11” on Page 14, Line 1). Please standardize terminology throughout the text.
- Abbreviations: Numerous abbreviations are used without definition upon first appearance, including ATAC, BAC, VSV, and others. Please ensure all abbreviations are defined when first introduced.
- Figure S9: The label “c” is missing and should be added.

Reviewer #2

(Remarks to the Author)

In this study, the authors demonstrate that the human placenta-specific microRNA cluster on chromosome 19 (C19MC) suppresses excessive innate immune responses in trophoblasts. They first identified regulatory enhancer elements of C19MC through ATAC-seq. Deletion of one such enhancer (ATAC-11) using CRISPR-Cas9 led to the loss of C19MC ncRNA expression and enhanced TLR3-dependent interferon responses in BeWo cells. These findings suggest that C19MC ncRNAs restrict TLR3 overactivation in trophoblasts. The experiments are well designed with appropriate controls, and the data interpretation is generally sound.

Specific comments:

1. Page 4, lines 13-14: A recent paper (ref. 28) reported the deletion of the C19MC promoter in hTSCs. The main findings of this paper should be mentioned in the introduction.
2. ATAC-11 deletion reduced proliferation in TSCs. Could the authors comment on whether C19MC ncRNAs influence proliferation in BeWo cells?
3. Page 7 line 21: The authors state that “using RNA-seq, we found marked upregulation of IFNs”. However, Fig. 3b does not contain IFNs. RNA-seq data of IFNs should be added to Fig. 3b.
4. This manuscript clearly demonstrates that C19MC suppresses TLR3-mediated sensitivity to poly(I:C). However, the underlying mechanism remains unclear. Fig. S8 shows that TLR3 is induced upon ATAC11 deletion. Is this upregulation a direct consequence of C19MC suppression? In addition, the authors propose that C19MC RNAs might bind to TLR3 and block its activation (Page 14, lines 26-27). According to page 15 ([add competition experiment]), the authors may plan to perform a competition assay between C19MC RNAs and poly(I:C), which, if performed, would greatly strengthen their proposed model.
5. Fig. 4b shows that suppression of C19MC miRNAs in ATAC11-deleted hTSCs is only partial. To properly interpret the data in Fig. 4, the efficiency of the ATAC-11 deletion should be assessed.
6. In Fig. 5b, it is unclear which samples are being compared to “Poly(I:C) Tfx”. Please clarify this comparison.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate the authors' efforts in revising the manuscript. I have no further comments.

Reviewer #2

(Remarks to the Author)

>Response to Comment3

Were the qPCR results in panels e-g reproduced by RNA-seq? Please consider including IFNs in Fig. 3b.

The authors have properly addressed my other concerns.

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The Chromosome 19 miRNA Cluster Guards Trophoblasts Against Overacting Innate Immunity

MS ID: COMMSBIO-25-5060-T

Corresponding author: Y Sadovsky

Response to reviewers

We thank the reviewers for their insightful comments and suggestions. Below are our responses and details of the additional experiments that we performed, and text modifications.

Reviewer 1:

In this study, Mouillet et al. explored the role of the chromosome 19 miRNA cluster (C19MC), a primate-specific placental microRNA cluster, in modulating innate immune responses. The authors first mapped trophoblast-specific accessible chromatin regions on chromosome 19 and proposed that these regions encompass enhancers regulating C19MC expression. They then performed CRISPR/Cas9-mediated deletions of selected elements in the trophoblast cell line BeWo to assess their functional relevance. Notably, BeWo cells lacking the ATAC-11 region (BeWo-dA11.21 and -dA11.23) exhibited resistance to viral infection and demonstrated spontaneous upregulation of interferons and interferon-stimulated genes (ISGs), which was further enhanced by exposure to synthetic double-stranded RNA, poly(I:C). Using diverse pathogen-associated molecular patterns and analyses of dsRNA localization, the authors provided evidence implicating Toll-like receptor 3 (TLR3) in this amplified innate immune response. They conclude that C19MC protects trophoblasts against excessive innate immune activation during viral infection. The findings are novel and relevant, significantly expanding our understanding of trophoblast biology.

Comment 1: Mechanistic Evidence and Role of C19MC miRNAs - While the experiments presented here clearly demonstrate that deletion of an enhancer region for C19MC in trophoblast cells leads to spontaneous activation of interferon-associated pathways and heightened responses to dsRNA, the mechanistic involvement of C19MC miRNAs themselves in regulating these immune responses remains insufficiently substantiated. Much of the proposed mechanism appears to be inferred from prior studies rather than conclusively established in this work. Given the mechanistic emphasis stated in the title of the manuscript, it would considerably strengthen the study if the authors could include experimental evidence directly supporting the role of C19MC miRNAs in these regulatory processes.

Response: Thank you for this important insight. As noted by the two reviewers, our data establish the role of C19MC miRNA in regulation of trophoblast innate immunity. While our data point to the attenuated activity of endosomal TLR3 in the presence of C19MC small RNAs, we have only been able to show that there were marked changes in the amount of endosomal TLR3 and higher levels of poly(I:C) in TLR3-containing endosomes in BeWo-dA11, when compared to BeWo-Cas9 cells (Fig. 6A-C). However, the strong induction of TLR3 expression in response to poly-IC (Fig. 4a for RNA and Figs. 6a, S8b-c, and S9a for protein) would render the results of any direct competition at the TLR3 level between C19MC RNAs and poly-IC uninterpretable. Further, considering the neutral cytoplasmic pH and the acidic endosomal pH, an in vitro competition would not have reliably captured the pH-dependent dsRNA binding (ref 38 in our text).

In response to both reviewers we conducted an RNA immunoprecipitation experiment in order to define which C19MC RNA species (miRNA, Alus, other species) might directly bind TLR3. Our results did not reveal direct enrichment of RNA binding to TLR3. Thus, while our

data show interaction and mitigation of innate immune signals at the level of the endosomes, we remain uncertain on which immune molecules, likely complexed with poly(I:C), may mediate this signal, and believe that the full elucidation of the interaction between C19MC RNAs and endosomal immune mediators may exceed the scope of the current work. We have expanded the limitation section in the discussion (page 24) and modified statements across our text to attest for this issue.

Comment 2: Specificity of dsRNA Receptor Involvement -The data indicating TLR3 involvement in the observed immune activation are compelling; however, the interpretation warrants some caution. The various dsRNA receptor ligands employed in these experiments are not entirely specific for individual dsRNA sensors and exhibit overlapping recognition profiles. Therefore, concluding that the responses are exclusively mediated by TLR3 may be an overstatement based solely on ligand stimulation. Additionally, although the co-localization of dsRNA with endosomes provides supportive information, it remains indirect and does not constitute definitive evidence of TLR3 engagement. The authors should consider moderating this interpretation or conducting additional experiments, such as knockdown of other dsRNA receptors like RIG-I or MDA5, to clarify receptor specificity.

Response: Thank you. As noted in our response to comment 1, we have indeed moderated our conclusions with regard to the specificity of the effect of TLR3. While our knockdown of TLR3 and the changes in the expression pattern of endosomal TLR3 are suggestive, they are not conclusive. Our observation regarding the marked upregulation of ISGs in C19MC-depleted cells rendered the manipulation of individual ISGs, including other dsRNA receptors, difficult to perform and to interpret. Please see also comment #1. As the reviewer intimated, there is also some cross talk among the endosomal and cytoplasmic pathways. Untangling these intricate relationships has not been our major goal in this project, and to be done properly will require major experimental endeavors. Note also that RIG-1 and MDA5 receptors are not endosomal, and the pathways we uncovered all point to an endosomal process (Fig. 5-6 and S9).

As recommended by the reviewer we have explained this weakness and moderated our interpretation in that context across the text and especially in the discussion (page 24).

Comment 3: Page 5, Line 10: The transition between the discussion of BAC plasmid sequences and the analysis of C19MC expression in primary trophoblasts is somewhat abrupt. Additional context would help improve the coherence and clarity of this section.

Response: As suggested by the reviewer, we edited this paragraph to add more context to our analysis of open chromatin sites (page 5, lines 88-90).

Comment 4: Page 5, Line 18: The rationale for focusing specifically on the autonomous enhancer activity of C19MC regions should be elaborated in more detail.

Response: We agree, this was not our focus, and we therefore removed the term “autonomous” from the text. While an autonomous enhancer could be relevant for driving placenta-specific transgene expression, the experiment described in this paper focused on identifying a sequence whose deletion suppresses C19MC expression. We thus aimed to identify a region necessary for expression, regardless of its autonomy. We also added a brief explanation clarifying the goal of these experiments. (page 6, lines 122-125)

Comment 5: Page 6, Lines 9–15: What evidence supports the assumption that ATAC peaks identified in human trophoblasts would demonstrate comparable activity in the mouse placenta? This requires clarification, particularly since C19MC is absent from the mouse genome.

Response: Data from our transgenic C19MC expressing mice, which we previously published (Chang et al. 2017, ref 17 in our revised text) showed that C19MC miRNA exhibited an expression pattern in mice similar to that in humans, with predominant expression in the pregnant dam's placenta. In this paper, we also compared the expression pattern of C19MC miRNA in primary human trophoblasts (PHTs) with that observed in the transgenic mice, and found a significant correlation between the two models further suggesting a shared regulatory mechanism.

Comment 6: Page 8, Lines 6–7: It is commendable that BeWo-dA3/4-21 and -41 were included as negative controls to exclude potential off-target effects arising from lentiviral infection. However, did the authors verify the successful deletion of the A3/4 regions in these two BeWo clones?

Response: We performed multiple PCR verifications on genomic DNA extracted from cells taken at different stages of the procedure to identify clones in which all the alleles were edited. Genomic DNA was extracted using guanidine thiocyanate followed by silica column purification. PCR primers were designed to flank the edited region, and the PCR conditions were optimized to detect the edited, partially edited, and non-edited amplicons. This approach yielded unambiguous PCR profiles, clearly distinguishable clones with full, partial, or no deletion of the targeted sequence. We added these details to the methods, on page 30, lines 602-605.

Comment 7: Page 14, Lines 18–20: The logical basis of this sentence is not fully articulated and should be further elaborated to clarify the argument.

Response: We appreciate this comment and apologize for the lack of clarity. We have revised this section (page 25, lines 469-473) to indicate “The immune system during pregnancy must balance a potent response to pathogens with the prevention of excessive activation by fetal alloantigens or other stimuli. Such immune activation has been implicated in a range of adverse neonatal outcomes^{51,52} (and reviewed in⁵³). The balancing signals may originate from the maternal immune system or from the fetal-placental unit.”

Minor Comments

- Page 5, Line 5: Please spell out “BAC” at its first mention.

Response: Thank you. Done (page 4, line 82)

- Page 5, Line 14: The word “cell” is unnecessary here, as HUVEC already stands for “Human Umbilical Vein Endothelial Cell.”

Response: Thank you. Cell removed (Page 5, now line 94)

- Page 15, Line 2: The phrase “[add competition experiment]” appears to have been inadvertently retained and should be removed.

Response: Our apologies. This inadvertent comment reflected our attempt to perform these experiments. Please see Comments 1 and 2, above.

- Nomenclature Consistency: The manuscript inconsistently refers to ATAC-11 (e.g., as “dd11” on Page 13, Line 25, and as “A11” on Page 14, Line 1). Please standardize terminology throughout the text.

Response: We apologize and have corrected these and a few other annotations across the text.

- Abbreviations: Numerous abbreviations are used without definition upon first appearance, including ATAC, BAC, VSV, and others. Please ensure all abbreviations are defined when first introduced.

Response: We apologize and have corrected these and a few other annotations across the text.

- Figure S9: The label “c” is missing and should be added.

Response: Thank you. This has been corrected – we referred to Fig. S9b, right and left panel.

Reviewer 2:

In this study, the authors demonstrate that the human placenta-specific microRNA cluster on chromosome 19 (C19MC) suppresses excessive innate immune responses in trophoblasts. They first identified regulatory enhancer elements of C19MC through ATAC-seq. Deletion of one such enhancer (ATAC-11) using CRISPR-Cas9 led to the loss of C19MC ncRNA expression and enhanced TLR3-dependent interferon responses in BeWo cells. These findings suggest that C19MC ncRNAs restrict TLR3 overactivation in trophoblasts. The experiments are well designed with appropriate controls, and the data interpretation is generally sound.

Comment 1: Page 4, lines 13-14, a recent paper (ref. 28) reported the deletion of the C19MC promoter in hTSCs. The main findings of this paper should be mentioned in the introduction.

Response: Thank you, and we agree. This has been added to page 4, now lines 70-73.

Comment 2: ATAC-11 deletion reduced proliferation in TSCs. Could the authors comment on whether C19MC ncRNAs influence proliferation in BeWo cells?

Response: We observed a slight reduction in the proliferation rate of the mutant BeWo cells; however, the reduction is negligible when compared to that of TSCs. Interestingly, in a previous study, we found that highly invasive extravillous trophoblasts (EVTs) in the placenta express lower levels of C19MC miRNAs compared to villous trophoblasts (Xie et al. 2014). In contrast, Kobayashi et al. (2022) observed that silencing C19MC miRNAs also led to a proliferation arrest in TSCs. Altogether, these results suggest that the C19MC role in proliferation is complex and probably context dependent.

Comment 3: Page 7 line 21, The authors state that “using RNA-seq, we found marked upregulation of IFNs”. However, Fig. 3b does not contain IFNs. RNA-seq data of IFNs should be added to Fig. 3b.

Response: Thank you for pointing this out. Our mistake. We have revised the sentence to “using RNA-seq, we found marked upregulation of interferon-stimulated genes (ISGs)” (now on page 10, line 203). The IFN data is shown a bit later, in panels e-g of the same figure.

Comment 4: This manuscript clearly demonstrates that C19MC suppresses TLR3-mediated sensitivity to poly(I:C). However, the underlying mechanism remains unclear. Fig. S8 shows that TLR3 is induced upon ATAC11 deletion. Is this upregulation a direct consequence of C19MC suppression? In addition, the authors propose that C19MC RNAs might bind to TLR3 and block its activation (Page 14, lines 26-27). According to page 15 ([add competition experiment]), the

authors may plan to perform a competition assay between C19MC RNAs and poly(I:C), which, if performed, would greatly strengthen their proposed model.

Response: Thank you for this important point. Please see also our Comment 1-2 to reviewer 1. Our inadvertent entry indeed reflected our plan to perform this competition experiment. However, we found that this experiment would be challenging to interpret because of the upregulation of TLR3, which would create an apparent increase in poly(I:C) binding in C19MC-depleted cells, but not because of decreased competition. As noted, in response to these comments we performed a RIPseq experiment but observed no difference in direct RNA binding to TLR3, suggesting a more intricate competition in endosomal complexes. We have modified the text, mainly on page 20, lines 380-381, and the limitation section on page 24, to emphasize these points.

5. Fig. 4b shows that suppression of C19MC miRNAs in ATAC11-deleted hTSCs is only partial. To properly interpret the data in Fig. 4, the efficiency of the ATAC-11 deletion should be assessed.

Response: Figure 4b presents a series of experiments involving CRISPR-Cas9 editing of TSCs. However, we did not isolate or select individual clones due to the difficulty in maintaining cells carrying deletion of the ATAC11 enhancer. This is explained on pages 13-14, lines 261-265. Instead, the downstream experiments were performed using a polyclonal population, which included both edited cells (confirmed by PCR on genomic DNA) and a substantial fraction of non-edited cells. Consequently, the observed effects are of much lower amplitude compared to those seen in experiments using clonal BeWo cell lines.

6. In Fig. 5b, it is unclear which samples are being compared to “Poly(I:C) Tfx”. Please clarify this comparison.

Response: We agree and apologize for the insufficient clarity. Our main goal was to show the effect of bafilomycin A1, an inhibitor of endosome acidification, on poly(I:C)-exposed WT and dA11 cells. We found that the high response of BeWo-dA11 cells to poly(I:C) was abrogated by bafilomycin treatment. We also tested the poly(I:C) delivery method. Previous studies have shown that cellular responses to poly(I:C) can vary significantly depending on whether it is administered freely in the culture medium or delivered through transfection. Therefore, we compared poly(I:C)-exposed cells to untreated controls and also evaluated the influence of bafilomycin in each condition. Interestingly, while transfection of poly(I:C) elicited a moderate response in BeWo-WT cells, the response in dA11 cells was weaker, in contrast to free poly(I:C). Each of the two experimental components was compared to its own control (orange bars). We have added a brief explanation to the legend of Fig. 5b.

The Chromosome 19 miRNA Cluster Guards Trophoblasts Against Overacting Innate Immunity

MS ID: COMMSBIO-25-5060A

Corresponding author: Y Sadovsky

Response to reviewers

We thank the reviewers for their insightful comments and suggestions. Below are our responses and details of the additional experiments that we performed, and text modifications.

Reviewer 1:

Response: Thank you for your supportive comment

Reviewer 2:

Were the qPCR results in panels e-g reproduced by RNA-seq? Please consider including IFNs in Fig. 3b.”

Response: As noted in the original legend of Figure 3, panel 3e was RT-qPCR, yet panels 3f-g were based on ELISA, designed to support the data obtained by RNAseq and by RT-qPCR. As requested by the reviewer, we reevaluated the expression of IFNs in our RNAseq data (Figure 3b). Only IFNL1 met our rigorous criteria for inclusion in Figure 3b (these criteria included being among the top ~100 genes for expression difference, and a detectable baseline expression.) IFNL2 and IFNB, which were tested in panels 3e and 3g, did not meet those criteria and therefore not included in Figure 3b. The new version of our paper does include IFNL1 in Figure 3b, as requested. We have also better aligned the Figure 3b clusters, for easier data visualization.