Physiologically relevant miRNAs in mammalian oocytes are rare and highly abundant.

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Transaction Report:

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As you will see, all referees acknowledge that the findings are interesting and novel. They only have some more minor suggestions for how the study could be improved and strengthened, and I think that all should be addressed. Please let me know in case you disagree so that we can discuss this further.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revisions.

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In a very focused and clear manuscript, Kataruka et al demonstrate that endogenous miRNAs can be active in oocytes, provided they reach sufficient concentration. They describe two such cases, mir-10a in Bos taurus and mir-205 in sus scrofa. This is very significant as the same group made a very convincing case that in general miRNA levels are simply too low in oocytes to allow meaningful miRNA activity in oocytes. These two cases now show that also in mammals, biologically relevant miRNA activity can be present in oocytes, even though it appears to be rather species specific.

Beyond showing that miRNAs can be active, the authors also show that likely miRNA stability is an important aspect for these specific miRNAs to reach such high abundance. However, it could not be pin-pointed where the stability derives from.
In general, I have very little to ask for, regarding this work. The text is clear, and the results I think merit publication in EMBO Reports. There are a few minor aspects that should be addressed though.

1) In general, I could not find how many biological replicates were tested in each case, and what sort of statistics were used to assess significance. This has to be provided. Assuming that at least 3 biological replicates are represented, this is merely a textual issue.

2) The stability argument was tested with only one unstable miRNA as control. It would make a stronger case if multiple control miRNAs could be tested, and maybe also some that are relatively abundant, even if not so abundant as mir-205.

3) On page 11, the authors refer to Figure 3E, which should be Figure 4.

4) Figure 4B does not seem to have any error bars, and it is also unclear what exactly ‘relative amount’ means. Is all scaled against non-treated? If so, it would still be good to provide the data of the PCR cycles. How consistent were these amongst replicates? Personally, a Northern blot would also be appreciated here, but I do not know whether sufficient oocytes can be collected for that.

5) It would be nice if the authors could check for 3’ end uridylation, or adenylation, in relation to stability. Do Bt-mir-10b and Ss-mir-205 show differential properties, compared to non-abundant miRNAs?

Referee #2:

Most miRNAs are present at low concentrations in oocytes given the large growth in volume of these cells and the fact that miRNA production and turnover are not adjusted to offset that growth. The consequence of this low concentration is that most miRNAs are not functional in oocytes/zygotes. Here, the authors report the existence of two abundant miRNAs that manage to accumulate at high, functional levels in grown porcine and bovine oocytes. Whereas most miRNAs achieve concentrations quite below 0.5 nM, mir-205 reaches ~4.4 nM in porcine oocytes and mir-10b, ~0.5 nM in bovine oocytes. These represent clear outliers when comparing to other miRNAs and to all miRNAs from the mouse.

The authors do two types of experiments to assess whether these miRNAs are functional: the inject reporter miRNAs into oocytes that either contain binding sites for the respective miRNAs or not and this shows that reporters with binding sites are repressed in both porcine and bovine oocytes. In the second experiment, the authors inject a miRNA inhibitor for miR-205 in porcine oocytes and observe that some endogenous mRNAs that are predicted to bind to miR-205 are derepressed. Interestingly, the treatment of porcine embryos with the mir-205 inhibitor results in embryonic arrest, suggesting that this miRNA may play a role in embryogenesis.

Finally, the authors try to address whether mir-205 reaches higher concentrations than other miRNAs due to increased transcription rates or increased stability. The authors argue that transcription is not particularly high, but that higher stability of mir-205 is what makes it so highly abundant. However, I find this section biased:
- The authors base most of their arguments that transcription is not particularly high on abundance of lncRNAs that host the miRNAs. However, steady-state abundance of lncRNAs is not a good proxy for the transcriptional rate of the pri-miRNAs. Not only do we not know the stabilities of these different lncRNAs but also, given that the production of mir-205 and the lncRNA are mutually exclusive this means that there is a large fraction of transcript that is likely not contributing to lncRNA steady-state abundance.
- The steady state abundance is the combination of both the rates of transcription and decay. One of the main arguments for the authors to say that decay plays a bigger role is that there is another miRNA cluster that produces similar levels of host lncRNA but lower levels of mature miRNAs. This is has to be reinterpreted given my points above, but in addition, it is highly probably that there are other miRNAs that are as stable as mir-205 but have a lower transcription rate and therefore accumulate at lower levels. Relative to such a miRNA, it would be transcription that makes the difference.
- Of course it would be a different story if the authors could measure rates of production and decay across many/all miRNAs, then they could possibly make some of the statements they make. I realize this is an extremely challenging experiment in this system and I am not suggesting that the authors need to do these measurements. But then this whole section should in my opinion be rewritten.

In addition to the point above, I have one additional relatively minor request:
For miRNA quantification, the authors use a qPCR-based strategy. It is not stated in the methods but I presume the authors measured a titration series of a synthetic miRNA to be able to estimate copy numbers. It would be important to provide all this raw data as a supplementary table.

Minor points:
- Fig. 1A and Fig. 4 has a transparency issue on the axes
Referee #3:

Current evidence suggests that the number of binding sites in a cell for a specific miRNA exceeds the number of molecules of that miRNA. Moreover, functional miRNAs are generally present at nM or high pM intracellular concentration, i.e., several orders of magnitude greater than the KD for binding of the miRNA to seed-matched sequences in target RNAs. Consequently, miRNAs generally follow a stoichiometric binding regime, in which each additional molecule of miRNA linearly increases target site occupancy, with greater occupancy of high affinity sites (e.g., 7mer-A1 or 7mer-m8 sites) than of the more abundant low affinity sites (e.g., 5mer sites). In this manuscript, Svoboda and colleagues identify two miRNAs—one in pig and one in cow—that are present in oocytes at sufficiently high concentrations to repress target gene expression. These two miRNAs represent the first maternally provided, mammalian miRNAs with a clear function in oocytes. This manuscript makes an important contribution to the field, but requires minor revisions before publication.

Main Concerns

(1) Page 6 ff: The analysis of site occupancy based on miRNA and site abundance is more sophisticated than most miRNA studies, but is not formally correct. The concentration of sites in the transcriptome, not the concentration of mRNA molecules, is what matters for mRNA repression by miRNAs. A miRNA will be distributed among sites based on the concentration of each site type, the affinity of each site type, and the concentration of the miRNA. Thus, what matters for a reporter with four bulged sites is the affinity of such sites for the miRNA, including any cooperative interactions for closely spaced sites, the site concentration (\(4 \times \) the mRNA concentration), and the affinity and concentration of each of the various site types. For a miRNA to regulate the reporter, the number of endogenous sites must be less than the number of miRNA molecules (leaving sufficient miRNAs to bind the reporter) and/or the affinities of the reporter sites must be sufficiently greater than those of the endogenous sites. Ideally, a globally fitted mathematical model or a simulation should be used. At the very least, the simplifying assumptions need to be discussed in more detail.

(2) Page 7: miRBase provides target predictions using TargetScanVert, miRDB, or microrna.org. What does it mean for a target to be "the best predicted...in miRBase"? This was not described in the methods (or I missed it). The false-positive rates for these three algorithms are quite different; if the authors took, for example, the intersection of the three algorithms, the targets are unlikely to be higher confidence than using TargetScan alone.

(3) Figure 1C: is the qRT-PCR method used sensitive to the precision of the miRNA 3′ end or to non-templated nucleotide addition?

Minor Concerns

(1) It is important to stress for the reader that bulged sites, first used by Doench and Sharp, are a convenient tool for reporter assays, but do not exist in nature.

(2) Page 3: the first demonstration that the extent of complementarity determines whether a miRNA cleaves a target RNA ("RNAi") or binds via limited complementarity and recruits components of mRNA turnover pathways (miRNA-like) was Hutvagner et al. (Science 2002).

(3) RNA-seq data can easily provide absolute molecular abundance when synthetic spike-in oligos are used, e.g., Gainetdinov et al. (Mol Cell 2018).

(4) Page 12: how can the hypothesis of Piwecka et al. be consistent with the stoichiometric model which the authors use to interpret their findings? Piwecka et al. seemed to me to be proposing that the loss of abundant seed-matched binding sites for a miRNA led to reduced miRNA abundance and increase target levels. This is hard to reconcile with the conventional view of stoichiometric binding.
Referee #1:

In general, I have very little to ask for, regarding this work. The text is clear, and the results I think merit publication in EMBO Reports. There are a few minor aspects that should be addressed though.

1) In general, I could not find how many biological replicates were tested in each case, and what sort of statistics were used to assess significance. This has to be provided. Assuming that at least 3 biological replicates are represented, this is merely a textual issue.

Indeed, experiments were performed in at least biological triplicates. An exception in the first submission was quantification of ssc-miR-10b, which was done as a technical triplicate on one cDNA sample (Fig. 1C). For the revised version, this analysis was completed as a biological triplicate – that’s why the S.s. sample in Fig. 1C bar differs from the first submission.

2) The stability argument was tested with only one unstable miRNA as control. It would make a stronger case if multiple control miRNAs could be tested, and maybe also some that are relatively abundant, even if not so abundant as mir-205.

We added analysis of two additional miRNAs, which were relatively abundant in porcine oocytes ($10^5$), and we had primers from previous analyses – miR-22 and miR-10b. These data were added into Fig. 3A and as an extended view figure EV4A. Both miR-22 and miR-10b are relatively abundant in porcine oocytes and are also less stable than miR-205 but more stable than let-7. A future systematic quantitative analysis of all maternal miRNAs by small RNA-seq will clarify half-lives of maternal miRNAs in oocytes of different mammals but is beyond the scope of the current manuscript.

3) On page 11, the authors refer to Figure 3E, which should be Figure 4.

The error was corrected.

4) Figure 4B does not seem to have any error bars, and it is also unclear what exactly ‘relative amount’ means. Is all scaled against non-treated? If so, it would still be good to provide the data of the PCR cycles. How consistent were these amongst replicates? Personally, a Northern blot would also be appreciated here, but I do not know whether sufficient oocytes can be collected for that.

Error bars were added, we overlooked we did not insert them, we apologize for that. As mentioned in methods, oxidation experiments were performed three times, each time in a triplicate PCR, consistency of replicates was good and there was typically a 6-7 cycle difference. “Relative amount” meant scaling treated to non-treated samples, which were set to one. Regarding a Northern blot from oocytes, it is not feasible to analyze absence of 2’-OH modification by Northern blotting.

5) It would be nice if the authors could check for 3’ end uridylation, or adenylation, in relation to stability. Do Bt-mir-10b and Ss-mir-205 show differential properties, compared to non-abundant miRNAs?

We analyzed uridylation and adenylation and added these data to the results section. Analysis of non-templated additions of five most abundant miRNAs in murine, bovine and porcine oocytes suggests that
*bta-miR-10b* and *ssc-miR-205* do not have unique properties in terms of non-templated 3’ additions. These data are provided as a new panel Fig. 4D. The main non-templated addition to *bta-miR-10b* and *ssc-miR-205* miRNAs is monoadenylation. This modification is common among maternal miRNAs (DOI: 10.1126/sciadv.1501482).

Among the top five most abundant porcine miRNAs, *ssc-miR-205* has higher monoadenylation (16.4%) than the other miRNAs (2.6% to 5.9%). However, association of monoadenylation with the observed miR-205a stability and abundance is elusive - is monoadenylation contributing to stability of *ssc-miR-205* or is it observed at higher frequency because miR-205 is more stable? Furthermore, we observed monoadenylation in bovine and porcine oocytes where it did not correspond to miRNA abundance: 4.5% of *bta-miR-10b* exhibit monoadenylation while it is over 19% for *bta-miR-92a*, which has ~4x less RNA-sequence reads than *bta-miR-10b*. Finally, high heterogeneity of the 3’ adenylated miRNA fraction (ranging from a few % to over 50%) was reported for murine maternal miRNAs and there was no apparent correlation between miRNA abundance and 3’ adenylation (see the graph below with abundance-ranked miRNAs from DOI: 10.1126/sciadv.1501482).
Referee #2:

The authors argue that transcription is not particularly high, but that higher stability of mir-205 is what makes it so highly abundant. However, I find this section biased:

- The authors base most of their arguments that transcription is not particularly high on abundance of lncRNAs that host the miRNAs. However, steady-state abundance of lncRNAs is not a good proxy for the transcriptional rate of the pri-miRNAs. Not only do we not know the stabilities of these different lncRNAs but also, given that the production of mir-205 and the lncRNA are mutually exclusive this means that there is a large fraction of transcript that is likely not contributing to lncRNA steady-state abundance.

We agree, that available expression data cannot be conclusively interpreted. Our aim was to discuss possible interpretations and explain why we think available RNA-seq data do not provide strong evidence for uniquely high expression of \( \text{miR-205} \) in pig oocytes.

The spliced lncRNA from the miR-205 host gene has low abundance for a maternal transcript and the transcriptional landscape in the locus seems rather mundane considering exceptional abundance of \( \text{miR-205} \), which is two or more orders of magnitude more abundant than other miRNAs. Comparison with RNA-seq data from the same locus in bovine oocyte shows that porcine oocytes likely have more transcription in the locus. However, difference between mature miR-205 levels in bovine and porcine oocytes is two orders of magnitude while long RNA-seq data imply that the difference between spliced lncRNA host transcripts could be \(~20\) fold (Fig 3). Naturally, there could be several alternative explanations of these numbers. The bottom line is that none of the features of the porcine miR-205 locus suggests that it is adapted for exceptional \( \text{miR-205} \) expression. This makes it less likely that exceptionally high transcription rate of \( \text{miR-205} \) would be the sole cause of exceptionally high abundance of ssc-miR-205. At the same time, none of features of the ssc-miR-205 locus is conclusive to rule it out and each argument can be challenged. In any case, to address reviewer concerns (including the following points), we revised this part of the discussion to clarify the reasoning and separation of data from interpretation and hypothesizing.

- The steady state abundance is the combination of both the rates of transcription and decay. One of the main arguments for the authors to say that decay plays a bigger role is that there is another miRNA cluster that produces similar levels of host lncRNA but lower levels of mature miRNAs. This is has to be reinterpreted given my points above, but in addition, it is highly probably that there are other miRNAs that are as stable as mir-205 but have a lower transcriptional rate and therefore accumulate at lower levels. Relative to such a miRNA, it would be transcription that makes the difference.

We agree with reviewer’s points that both factors likely contribute to the high level of \( \text{miR-205} \). The text was revised to propose that transcription and other factors are likely responsible for high \( \text{miR-205} \) abundance in porcine oocytes.

We would like to point out that, in comparison to the traditional steady-state situation concerning transcript levels in somatic cells, transcripts in oocytes are generally highly stable while fully-grown GV oocytes become transcriptionally quiescent. Hence maternal transcript levels in fully-grown GV oocytes usually reflect accumulation of the bulk of the transcriptome during the growth phase (transcription and usually minimal mRNA decay) while culture of GV oocytes can reveal transcript turnover (only decay, no transcription).
-Of course it would be a different story if the authors could measure rates of production and decay across many/all miRNAs, then they could possibly make some of the statements they make. I realize this is an extremely challenging experiment in this system and I am not suggesting that the authors need to do these measurements. But then this whole section should in my opinion be rewritten.

We revised the criticized section. We agree that future analysis or rates of decay of all maternal mRNAs by RNA-seq will clarify the question of miRNA turnover rates but it is beyond the scope of the current work.

In addition to the point above, I have one additional relatively minor request:

For miRNA quantification, the authors use a qPCR-based strategy. It is not stated in the methods but I presume the authors measured a titration series of a synthetic miRNA to be able to estimate copy numbers. It would be important to provide all this raw data as a supplementary table.

For miRNA quantification, we used let-7 oligonucleotide-based calibration curve as described previously (doi: 10.1093/nar/gkaa543). In addition to the NAR manuscript, we produced an additional let-7-oligo-based calibration curve for transcripts > 1x10^6 molecules. To formally demonstrate that extremely high abundance of ssc-miR-205 is not an artifact of calibration/qPCR analysis, we generated also a calibration curve with miR-205 oligonucleotide and use that calibration curve for quantifying miR-205 amplification with the same primers (Figure EV1, new panels B (calibration curve) and new panel C (miR-205 estimates)). These new data also show exceptionally high miR-205 abundance, even higher when compared to the let-7-based calibration curve.

To address the reviewer’s question, we revised the manuscript and, in addition to RNA-seq data and let-7 calibration curves published previously, we add calibration curves for estimating highly abundant miRNAs (Figure EV1, panel B). We also provide reanalysis of miR-205 abundance with the miR-205 calibration curve (Fig. EV2, panel B), which suggests slightly higher miR-205 amount in porcine oocytes (close to 2x10^6 miRNA molecules per oocyte).

Minor points:
- Fig. 1A and Fig. 4 has a transparency issue on the axes

We revised description of axis labels in these figures.

- Line 216 refers to Fig. 3E but there is no such panel

This issue was corrected.
Referee #3:

Current evidence suggests that the number of binding sites in a cell for a specific miRNA exceeds the number of molecules of that miRNA. Moreover, functional miRNAs are generally present at nM or high pM intracellular concentration, i.e., several orders of magnitude greater than the KD for binding of the miRNA to seed-matched sequences in target RNAs. Consequently, miRNAs generally follow a stoichiometric binding regime, in which each additional molecule of miRNA linearly increases target site occupancy, with greater occupancy of high affinity sites (e.g., 7mer-A1 or 7mer-m8 sites) than of the more abundant low affinity sites (e.g., 5mer sites). In this manuscript, Svoboda and colleagues identify two miRNAs— one in pig and one in cow—that are present in oocytes at sufficiently high concentrations to repress target gene expression. These two miRNAs represent the first maternally provided, mammalian miRNAs with a clear function in oocytes. This manuscript makes an important contribution to the field, but requires minor revisions before publication.

Main Concerns

(1) Page 6 ff: The analysis of site occupancy based on miRNA and site abundance is more sophisticated than most miRNA studies, but is not formally correct. The concentration of sites in the transcriptome, not the concentration of mRNA molecules, is what matters for mRNA repression by miRNAs. A miRNA will be distributed among sites based on the concentration of each site type, the affinity of each site type, and the concentration of the miRNA. Thus, what matters for a reporter with four bulged sites is the affinity of such sites for the miRNA, including any cooperative interactions for closely spaced sites, the site concentration (4 × the mRNA concentration), and the affinity and concentration of each of the various site types. For a miRNA to regulate the reporter, the number of endogenous sites must be less than the number of miRNA molecules (leaving sufficient miRNAs to bind the reporter) and/or the affinities of the reporter sites must be sufficiently greater than those of the endogenous sites. Ideally, a globally fitted mathematical model or a simulation should be used. At the very least, the simplifying assumptions need to be discussed in more detail.

We agree that we simplified complexity of miRNA-mediated repression in order to highlight key features of the model system and experimental set ups. Our main point about concentration of mRNA molecules in the oocyte is that the oocyte has transcript density similar to that of somatic cells, i.e. what is usually presented as accumulation of maternal mRNAs can be also seen as maintenance of cytoplasmic mRNA concentration. Of course, cytoplasmic concentrations of binding sites would vary for each miRNA and cell type according to cell-specific gene expression patterns. However, it is safe to assume that, without a strong negative or positive selection, cytoplasmic concentrations of most miRNA binding sites would not show high fold (an order of magnitude) differences. For example, below are rank-sorted all hexamer frequencies calculated from RNA-seq data (i.e. taking into account also transcript abundance) from mouse ESCs and mouse and porcine oocytes. Highlighted are poly(A) motif and motifs complementary to seeds of miR-290 cluster (ESC-specific highly abundant family), miR-122, and miR-1. Most abundant motifs are mainly repetitive motifs and some A/U-rich motifs. Distribution of the bulk of the hexamers is in a range of low fold-changes suggesting that concentrations of miRNA binding sites in different cells would be generally proportional to mRNA content. Of course a refined analysis may identify statistically highly significant differences of binding site concentration between cell types, which would reflect adaptations of transcriptome composition in response to a highly abundant miRNA – however, this takes place at much finer scale than we are discussing in the paper where we consider two orders of magnitude
differences in volumes and common miRNA concentrations in oocytes and use reporters primarily to show that endogenous maternal miRNAs are able to repress them.

The argument about miRNA and mRNA concentrations we brought up on page 6 concerned orders of magnitude differences in concentrations of miRNAs and their targets and situations when repression of a reporter with bulged sites is not observed and when it is. Also, the comment on NanoLuc reporter amount was meant in the context of previous reporters, which required 10x higher amount of mRNAs for quantifying reporter activity. In any case, to address reviewer’s point, we revised the text to clarify generalizations.
(2) Page 7: miRBase provides target predictions using TargetScanVert, miRDB, or microrna.org. What does it mean for a target to be “the best predicted...in miRBase”? This was not described in the methods (or I missed it). The false-positive rates for these three algorithms are quite different; if the authors took, for example, the intersection of the three algorithms, the targets are unlikely to be higher confidence than using TargetScan alone.

We apologize for inadequate explanation. Target selection combined two strategies. For initial target testing, we selected two top-scoring mmu-miR-205 targets (prediction for ssc-miR-205 was not available) by TargetScanVert: Plcb1 and Rgs18 as porcine transcripts carried one conserved 8-mer binding site and additional 6-mers (Plcb1 had one and Rgs8 four in pig). However, Rgs8 transcript was not reliably detectable in porcine oocytes by qPCR and was excluded from the analysis. For remaining putative targets, we directly analyzed complementary seed sequences in 3’UTRs porcine transcripts and transcript abundancies in porcine oocytes. We used sequences of 3’UTRs of all ENSEMBL-annotated genes and counted presence of complementary sequences to ssc-mir-205 seeds (6mer UGAAGG, 7mer_m8 AUGAAGG, 8mer AUGAAGGA, and 7mer_1a UGAAGGA – similarly to other miRNA target prediction tools). From the top-scoring genes with most seed matches, we selected the additional four targets, which had low but well detectable expression in RNA-seq data from porcine oocytes (1-4 FPKM). The rationale for this criterion was real targets would be expected to have low expression. Each of the selected targets had 7-9 6mers and 2-4 7mers. We revised the methods section in the resubmitted manuscript to accurately describe putative target selection and properties of the putative targets.

(3) Figure 1C: is the qRT-PCR method used sensitive to the precision of the miRNA 3’ end or to non-templated nucleotide addition?

Non-templated additions should not affect qRT-PCR method as the prevailing non-templated nucleotide addition is monoadenylation and this would be indistinguishable from 3’ adenylation (see the added panel 4D), which is used in the protocol.

Minor Concerns

(1) It is important to stress for the reader that bulged sites, first used by Doench and Sharp, are a convenient tool for reporter assays, but do not exist in nature.

We revised the sentence referring to bulged reporters and determining the cleavage; reference to Doench, 2002 was added. The reference to Schmitter et al. was selected because in this publication targeted reporters were analyzed by 3’RACE and perfect reporters showed canonical RNAi-like cleavage in the middle of the perfect complementarity with a miRNA.

(2) Page 3: the first demonstration that the extent of complementarity determines whether a miRNA cleaves a target RNA (“RNAi) or binds via limited complementarity and recruits components of mRNA turnover pathways (miRNA-like) was Hutvagner et al. (Science 2002).

We added this reference, we kept Yekta et al. as they reported the first endogenous animal miRNA-mediated RNA-like cleavage.
(3) RNA-seq data can easily provide absolute molecular abundance when synthetic spike-in oligos are used, e.g., Gainetdinov et al. (Mol Cell 2018).

This is true in general, but it does not apply to the specific context of the statement to which this comment appears to refer to:

*Since RNA-seq data provided only relative estimates of miRNA abundance, we used quantitative RT-PCR to determine copy numbers per oocyte (Fig. 1C)*

We referred to published RNA-seq data from porcine and bovine oocytes, which were not spiked. Furthermore, even if those RNA-seq libraries would be spiked, qPCR validation of high abundance of specific miRNAs would be needed to rule out artifacts. Spike-in oligos allow to generate a calibration curve for RPM values but reliability of absolute molecular abundance of specific miRNAs depends on accurate conversion of the endogenous small RNA population into a sequencing library. Depending on the method used to produce the library, especially if it involves linker ligation to a small RNA, various artifacts concerning specific miRNAs may appear in the sequencing library, which cannot be remedied by the use of spikes.

(4) Page 12: how can the hypothesis of Piwecka et al. be consistent with the stoichiometric model which the authors use to interpret their findings? Piwecka et al. seemed to me to be proposing that the loss of abundant seed-matched binding sites for a miRNA led to reduced miRNA abundance and increase target levels. This is hard to reconcile with the conventional view of stoichiometric binding.

In our view, these could be two separated processes – we interpret Piwecka et al. work as an example of a miRNA-bound substrate, which can mediate miRNA accumulation. Cdr1 can be seen as a unique miRNA-binding substrate, which is resistant to miRNA-mediated decay and which enables miR-7 accumulation. Consequently, loss of Cdr1 results in reduced miR-7 levels and reduced repression of miR-7 targets.

A hypothetical target-mediated miRNA accumulation could be a unique evolutionary adaptation, which could explain why ssc-miR-205 accumulation is so selective. Since we observe miR-205-mediated repression in fully-grown oocytes, which demonstrates there’s enough available miR-205 to recognize and efficiently suppress the reporter, there could be two possible explanations – (i) the miRNA bound & stabilizing substrate would be present mainly during the growth phase or (ii) there is a favorable on/off rate of the miR-205 interaction with a miRNA bound substrate, which would result in increased ssc-miR-205 half-life and would help ssc-miR-205 accumulation. Oocytes represent a unique case for RNA metabolism where pol II transcripts have extended half-life and can be deadenylated without degradation.
Dear Petr,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees, and I am happy to say that all support its publication now. The referees only still have a few minor comments that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few other editorial changes are also required:

- Please add a Data Availability Section (DAS) to the end of the MAterials and MEthods. If you have not deposited any data in public databases please add a sentence to the DAS that explains it.

- Fig EV3B callout is missing. Fig EV4 panel callouts are missing. Please add.

- The EV tables should be uploaded individually using the file type Expanded View.

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https://embor.msubmit.net/cgi-bin/main.plex

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have carefully addressed the raised issues. The manuscript can be accepted as is in my opinion. A very nice and interesting contribution to the miRNA and oogenesis fields.

Referee #2:

The authors have satisfactorily addressed my initial concerns.

A couple of minor points:
1. I am glad I asked about the calibration curves, because for every miRNA there should be a separate calibration curve. Given that the miRNA sequences and the primer sequences are different in each case, it is expected that the calibration curves will look different for different miRNAs.
2. With regards to a response to another reviewer, it is indeed possible to see 2'OH modification by Northern, following oxidation, beta elimination and high resolution PAGE.
3. The following sentence in p. 7 needs to be reworded:
   It is important that analysis of miRNAs in oocytes and zygotes would address and respect physiological concentrations of miRNAs and their targets as experimental conditions beyond physiological ranges would increase the risk of producing non-physiological artifacts.

Referee #3:

The revised manuscript is ready for publication!
One personal quibble: In their response to Reviewer 1, the authors write, "it is not feasible to analyze absence of 2'-OH modification by Northern blotting." There are many published examples of using Northern blots to determine the 2'-OH status of small RNA. One example is Figure 6A in Vagin et al. (Science 2006).
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A couple of minor points:

1. I am glad I asked about the calibration curves, because for every miRNA there should be a separate calibration curve. Given that the miRNA sequences and the primer sequences are different in each case, it is expected that the calibration curves will look different for different miRNAs.

We agree that primer efficiency may differ in each case, and give a different calibration curve, so it was indeed important to demonstrate that miR-205 estimation using the let-7 oligonucleotide-based calibration was not an experimental artifact. At the same time, all three calibration curves for three different miRNA oligonucleotides that we produced for miRNA quantification experiments in oocytes were comparable and produced similar results.

2. With regards to a response to another reviewer, it is indeed possible to see 2'OH modification by Northern, following oxidation, beta elimination and high resolution PAGE.

Our comment regarding feasibility concerned availability of the slaughterhouse oocyte material needed for such an experiment, not the procedure itself. An oocyte may contain ~0.5 ng of total RNA (mouse oocyte estimate), for a northern for miR-205 and a control (which would be a much less abundant miRNA), we estimate we would need ~500-600 oocytes per lane, and a minimum of 1000 porcine oocytes per Northern blot, which is possible to obtain but not feasible considering the rarity of the material - collection of mouse oocytes can be scaled up, collection of porcine oocytes depends on the situation in the slaughterhouse and oocytes while several research groups need to share available oocytes.

3. The following sentence in p. 7 needs to be reworded: It is important that analysis of miRNAs in oocytes and zygotes would address and respect physiological concentrations of miRNAs and their targets as experimental conditions beyond physiological ranges would increase the risk of producing non-physiological artifacts.

The sentence was changed to: It is important that analysis of miRNAs would respect physiological concentrations of miRNAs and their targets in order to avoid artifacts generated by non-physiological ones.

Referee #3:

The revised manuscript is ready for publication!
One personal quibble: In their response to Reviewer 1, the authors write, "it is not feasible to analyze absence of 2’-OH modification by Northern blotting." There are many published examples of using Northern blots to determine the 2’-OH status of small RNA. One example is Figure 6A in Vagin et al. (Science 2006).

We are sorry, our statement did not mean that Northern blotting cannot be used to detect the 2’OH status of a miRNA, we meant technical issues with collecting material needed for such an experiment. In other words, the experiment is not an impossible one, but it was not feasible to perform it on porcine maternal miRNAs. In the Vagin et al. paper, they used 1-10 µg of total RNA from whole Drosophila ovaries. A mammalian oocyte contains ~0.5 ng of total RNA but the relative miRNA fraction in that amount is much smaller (~50x) than in total RNA from somatic mammalian cells. We estimate we would need 500-600 oocytes/lane as we used that for two Northerns from mouse oocytes in the past when we analyzed let-7 abundance. However, our porcine oocytes are obtained in limited amounts from a single slaughterhouse once a week (if and how many pigs they slaughter on that day), so the material is not as easily scaled up as oocytes from mice. While ssc-miR-205 might be detectable in 50 or so porcine oocytes, other miRNAs, which should be tested in parallel, are much less abundant – this is why we estimate we would need at least 500-600 oocytes per lane, which would make it 1000-1200 oocytes for a single Northern with two lanes (control and treated sample). That amount of oocytes would take about two months to collect if everything would go well, we would not need to run other experiments, and would not share the source of porcine oocytes with other laboratories.
Dear Petr,

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Best wishes,

Esther

Esther Schnapp, PhD
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The data shown in figures should satisfy the following conditions:

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Each figure caption should contain the following information, for each panel where they are relevant:

- A specification of the experimental system investigated (e.g. cell line, species name).
- The assay(s) and method(s) used to carry out the reported observations and measurements.
- An explicit mention of the biological and chemical entity(ies) that are being measured.
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- The exact sample size (n) for each experimental group condition, given as a number, not a range.
- A description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- A statement of how many times the experiment shown was independently replicated in the laboratory.
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  - Are tests one-sided or two-sided?
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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Please fill out these boxes. [Do not worry if you cannot see all your text once you press return]

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No antibodies were used.

9. Identify the source of all cell lines and report if they were recently authenticated (e.g., by SRB profiling) and tested for mycoplasma contamination.

No cell lines were used.

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11. Identify the committee(s) approving the study protocol.

Not applicable

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

Not applicable

13. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

The use of slaughterhouse material for collection of oocytes and their in vitro culture does not require ethical committee approval according to the Czech law.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

Not applicable

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Data are provided in the manuscript, there were no data generated in the study, which would require deposition.

19. Data deposition in a public repository is mandatory for:
   a. Protein, DNA and RNA sequences
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Data are provided in the manuscript, there were no data generated in the study, which would require deposition.

20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).

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