nature portfolio

Peer Review File

Schistosoma sex-biased microRNAs regulate ovarian development and egg production by targeting Wnt signaling pathway

Corresponding Author: Professor Guofeng Cheng

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)
Review: COMMSBIO-24-2346
“Schistosoma sex-biased microRNAs regulate ovarian development and egg production by 2 targeting Wnt signaling pathway”

Du, Xia & Li et al. present a tour de force which is impressive and could warrant publication in Communications Biology after some points are clarified

MAJOR comments

- The effect of inhibition is very strong with several thousand protein coding genes affected and I am skeptical about this as such a global shift seems unexpected for 1 microRNA. GO enrichment analyses seem unwarranted as so many genes are deregulated. My questions: how did you normalize between treatment / Control? How high was the concentration of inhibitors in the cells and can you exclude off-target effects?
- microRNA targets in 3D are not convincing. microRNAs typically interact via their 7 nt long seed sequence at position 2-8. Only two sites in Frizzled 7 look genuine. Question: did you clone 5,7,9 sites as shown in Figure 3d for reported assay?
- Recently, a range of platyhelminth genomes became available and I would like to ask the authors to confirm Mir-1989 and bantam target sites in frizzled (7) orthologues.

MINOR comments

- Figure 2 a,b are way too small.
- MIR-1989 is a Platytrochozoan specific microRNA 1
- Figure 1a,b: add header and sequences from MirGeneDB 1
- L83 sentence ends weirdly.

1. Fromm, B. et al. MirGeneDB 2.1: toward a complete sampling of all major animal phyla. Nucleic Acids Res. 50, D204–D210 (2022).

Reviewer #3

(Remarks to the Author)
This manuscript is well-designed and the experiments well-executed. The work builds on their previous publication that the

miRNAs Bantam miRNA and miR-1989 were related to ovarian development. This study is important as miRNAs such as Bantam miRNA and miR-1989 may be related to ovarian development and egg production. The schistosome eggs are responsible for pathogenesis and transmission of schistosomiasis. Understanding the molecular mechanisms of ovary development and egg production is important for identifying potential targets for intervention. As these miRNAs are invertebrate-specific, it makes them potentially better effective targets against schistosomiasis The current study demonstrates regulatory mechanisms for Bantam and miR-1989 in ovarian development and egg production in Schistosoma. Using siRNA, they demonstrated that Bantam and miR-1989 miRNA inhibition led to the regulation of mTOR-, Wnt- and Hippo signaling pathways. By examining the function of Bantam and miR-1989, they demonstrated that Schistosoma Frizzled proteins (Frizzled-5/7/9) were targeted by Bantam and miR-1989.
By using siRNAs, they showed that SjFrizzled-5/7/9 inhibition resulted in decreased egg production and altered ovarian architecture. Further studies using Yeast two-hybrid system with Frizzled-7 as bait revealed that Frizzled-7 may coordinate with 23 proteins to implement various regulatory functions. In particular they found that Frizzled-7 strongly interacts with SjRho to potentially regulate egg production and ovarian development. They went on to show that Bantam/miR-1989 mediated Wnt signal pathway is critical for regulating ovarian development and egg production in Schistosoma. Since Frizzled-7 is expressed in ovaries and SjRho is shown to interact with Frizzled-7, they first demonstrated that SjRho was highly expressed in the ovaries of female worms. They then demonstrated in an animal model of S. japonicum that treating infected mice with siSjRho resulted in reduced egg production and worm burden thus providing the proof of principle for a role for Bantam miRNA and miR-1989, Wnt signaling pathway, Frizzled-7 and Rho in S. japonicum ovary development and egg production.
The results will be useful for the schistosomiasis field as they add to the biology of schistosome reproductive development. The results such as the YTH studies offer some new paths for study. They also will be of interest to scientists that study other trematodes of medical and veterinary importance in the
Platyhelminths as their ovary development and egg production may use some of the same pathways.
One concern is that as one reads the results without putting some methods such as below the name of the inhibitor or the method used to inhibit, the reader has to go to the methods to understand how the result was obtained.
See attached word file for minor corrections.
Line103; identify the inhibitor
Line 107 identify the control inhibitor
Line 216; rewrite in ovarian part

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am happy with the answers and glad the authors resequenced. Are the new data on SRA?

In response to Reviewer #1

Review: COMMSBIO-24-2346

"Schistosoma sex-biased microRNAs regulate ovarian development and egg production by 2 targeting Wnt signaling pathway"

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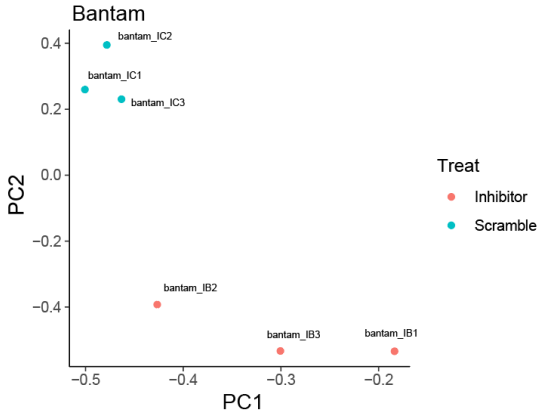
MAJOR comments

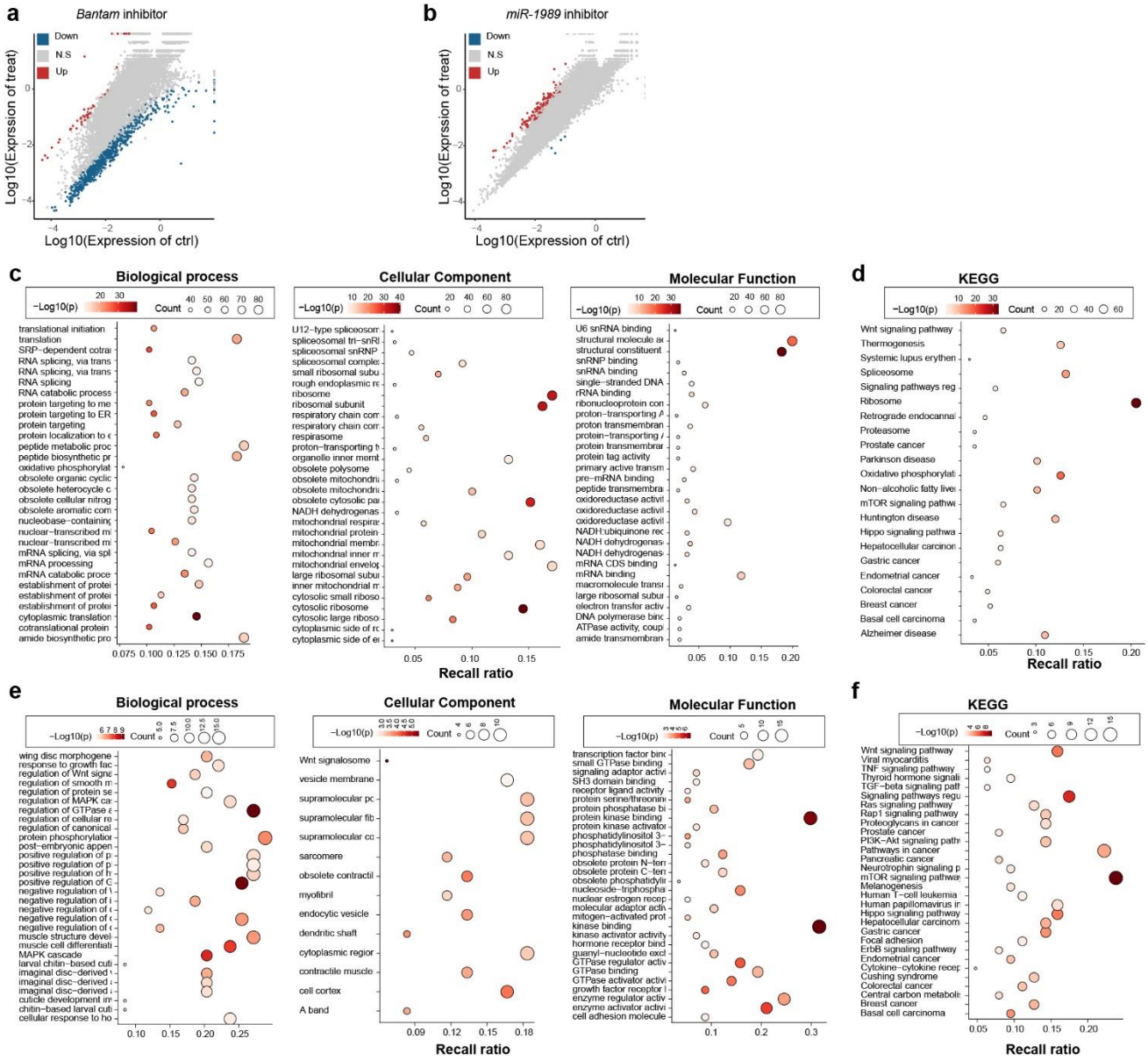
1."The effect of inhibition is very strong with several thousand protein coding genes affected and I am skeptical about this as such a global shift seems unexpected for 1 microRNA. GO enrichment analyses seem unwarranted as so many genes are deregulated. My questions: how did you normalize between treatment / Control? How high was the concentration of inhibitors in the cells and can you exclude off-target effects?"

Author Response: Following the constitutive suggestion from the reviewer, we checked raw data and PCA results for both *Bantam* and *miR-1989* inhibition results from RNA seq. We observed that within-group variation of all 6 samples from *bantam* inhibition, especially in samples of bantam_IB1 and bantam_IC1, implying that there may be something wrong during RNA seq analysis. Therefore, we re-sequenced these two samples (bantam_IB1 and bantam_IC1) using previous backup RNAs. PCA analysis of these two new results combined with previous 4 sample results indicated a considerable repeatability (Figure below). Then,



we preformed differentially expressed genes (DEGs) analysis using the updated data. We identified 780 DEGs after *Bantam* knockdown in parasites. Meanwhile, we found that the most top significant DEGs were repeatable in the updated data, including 93% DEGs (14/15) in Fig.2g (validated by real-time PCR), and *Fizzled5/7/9* in Fig.3e. Besides, all the functional enrichment analysis using DEGs were updated in revised manuscript (including Fig.2a-f and 3a-c), and we also observed that Wnt, mTOR, hippo, etc signaling pathway were significantly altered, which is consistent with our previous findings. Overall, the conclusion of our study is robust. Similarly, the repeatable results were also observed in *miR-1989* inhibition experiments.

The below figure is Fig 2a-f in the revised manuscript:



The below figure is Fig 3a-c in the revised manuscript:

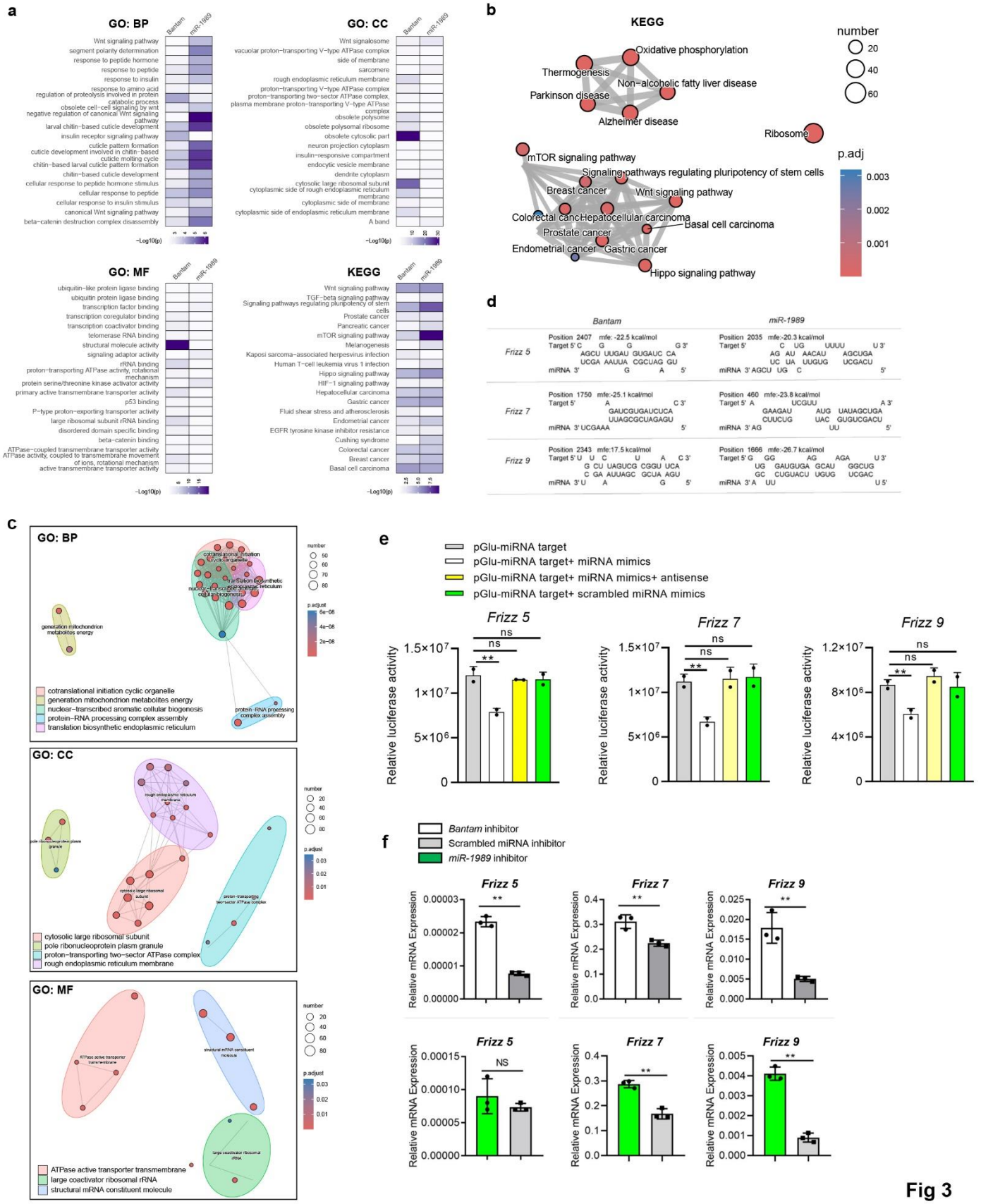


Fig 3

As to the analysis of differentially expressed genes between treatment and control, we used DEseq2 R package, a widely-used tool to estimate variance-mean dependence in count data from high-throughput sequencing assays based negative binomial distribution model (PMID: 20979621). DEGs were defined by fold change ≥ 2 and adjusted p-value ≤ 0.001.

For miRNA inhibition, we used 3 μg miRNA inhibitor per experiment (Line:481). The applied dosage had been optimized and used in our lab (PMID: 26871705). Following the identification of DEGs by RNA seq, we also validated the DEGs by RT-qPCR in independently prepared inhibition experiments. The identified miRNA targets were also validated by luciferase assay in mammalian cells. Consequently, our results indicated the identified targets for *Bantam* miRNA and *miR-1989* did not cause by off-target effect, at least *Frizzled-5/7/9*.

2." microRNA targets in 3D are not convincing. microRNAs typically interact via their 7 nt long seed sequence at position 2-8. Only two sites in Frizzled 7 look genuine. Question: did you clone 5,7,9 sites as shown in Figure 3d for reported assay?"

Author response: Yes, we cloned the putative target sites of *Frizzled 5/7/9* into a luciferase reporter vector. The recombinant plasmids were transfected into HEK 293T cells. At 24 h post-transfection, a control miRNA or miRNA mimics transfected into these cells. At 24-48 h post-transfection, luciferase activity was evaluated using a luciferase reporter assay system with normalization as protein assay. The results were shown in Fig 3e. The decreased luciferase activities upon the miRNA mimics transfection indicated *Bantam* miRNA could target the putative sites.

3." Recently, a range of platyhelminth genomes became available and I would like to ask the authors to confirm Mir-1989 and bantam target sites in frizzled (7) orthologues."

Author response: Based on the reviewer's comment, we used BLAST to analyze the homologs of *S. japonicum* Frizzled 5/7/9. After selection of top hit mRNAs, we used RNA hybrid to analyze the potential sites that target by *Bantam* miRNA and *miR-1989*.

The results were summarized in the following:

Names	IDs	Species	Target site prediction
Frizzled 7	XM_051210986.1	*Schistosoma haematobium*	miRNA : bantam length: 19 mfe: -20.3 kcal/mol p-value: undefined position 659 target 5' C GUG U 3' GCUU GAUCG GUCUC CGAA UUAGC UAGAG miRNA 3' U A GC U 5' miRNA : miR-1989 length: 22 mfe: -20.9 kcal/mol p-value: undefined position 2017 target 5' G ACAAACAAACUGUUU A 3' CGAA GACG GAAUACGGU GCUU CUGU CUUGUGUCG miRNA 3' A A ACU 5'
Frizzled 7	XM_024496394.1	*Echinococcus granulosus*	miRNA : bantam length: 19 mfe: -18.1 kcal/mol p-value: undefined position 349 target 5' G CCAUCA GU G 3' AGC UGCGAUC UCA UCG GCGCUAG AGU miRNA 3' AAAUUA 5'

			length: 1980 miRNA : miR-1989 length: 22 mfe: -23.8 kcal/mol p-value: undefined position 1418 target 5' G UGCAGGCUCCUCCAA UAUU C 3' GAAGAU GUG GCACAGUUG CUUCUG UAC UGUGUCGAC miRNA 3' AG U U 5'
Frizzled 5	XM_051217945.1	*Schistosoma haematobium*	miRNA : bantam length: 19 mfe: -18.8 kcal/mol p-value: undefined position 2471 target 5' A AAAC AUC U 3' AGC UAAUC CGAUUUCA UCG AUUAG GCUAGAGU miRNA 3' AA C 5' miRNA : miR-1989 length: 22 mfe: -26.7 kcal/mol p-value: 1.000000e+00 position 460 target 5' A UCGU UG A 3' GAAGAU UG UAUAGCUGA CUUCUG AC GUGUCGACU miRNA 3' AG U UU 5'
Frizzled 5	XM_024496394.1	*Echinococcus granulosus*	miRNA : bantam length: 19 mfe: -18.1 kcal/mol p-value: 1.000000e+00 position 349 target 5' G CCAUCA GU G 3' AGC UGCGAUC UCA UCG GCGCUAG AGU miRNA 3' AAAUUA 5' miRNA : miR-1989 length: 22 mfe: -23.8 kcal/mol p-value: 1.000000e+00 position 1418 target 5' G UGCAGGCUCCUCCAA UAUU C 3' GAAGAU GUG GCACAGUUG CUUCUG UAC UGUGUCGAC miRNA 3' AG U U 5'
Frizzled 9	XM_009174289.1	*Opisthorchis viverrini*	miRNA : bantam length: 19 mfe: -18.7 kcal/mol p-value: 1.000000e+00 position 1229 target 5' C GAUGC G G 3' GGCU U GCGGUUUU UCGA A CGCUAGAG miRNA 3' AAUU G U 5' miRNA : miR-1989 length: 22 mfe: -25.6 kcal/mol p-value: undefined position 790 target 5' A AUCU CACUG U 3' UUGGAGGCA GC ACGGCUGA AGCUUCUGU UG UGUCGACU miRNA 3' ACU 5'
Frizzled 9	XM_024491167.1	*Echinococcus granulosus*	miRNA : bantam length: 19 mfe: -22.9 kcal/mol p-value: undefined position 2463 target 5' G GG CU U 3' AGCU AAUCGUG GUCUU UCGA UUAGCGC UAGAG miRNA 3' AA U 5'

			miRNA : miR-1989 length: 22 mfe: -26.6 kcal/mol p-value: undefined position 2899 target 5' U AUUCCAA UG U U C 3' UCGG AG GUGA GCG CAGCUGG AGCU UC UACU UGU GUCGACU miRNA 3' UG 5'
Frizzled 9	XM_009170735	*Opisthorchis viverrini*	miRNA : bantam length: 19 mfe: -21.5 kcal/mol p-value: 1.000000e+00 position 942 target 5' G ACAU AG U 3' GGC G GCGAUCUC UCG U CGCUAGAG miRNA 3' AAAU AG U 5' miRNA : miR-1989 length: 22 mfe: -22.6 kcal/mol p-value: 1.000000e+00 position 1321 target 5' G A UUACA U 3' UCGGAGA AUGGAUGC UGG AGCUUCU UACUUGUG ACU miRNA 3' G UCG 5'

4. *"MINOR comments*

- Figure 2 a,b are way too small.

- MIR-1989 is a Platytrochozoan specific microRNA 1

- Figure 1a,b: add header and sequences from MirGeneDB 1

- L83 sentence ends weirdly.

1. Fromm, B. et al. MirGeneDB 2.1: toward a complete sampling of all major animal phyla. Nucleic Acids Res. 50, D204–D210 (2022)."

Author response:

-In the revised manuscript, we updated Fig 2a and 2b using the large ones.

-We included the header in Fig 1a and 1b in revised manuscript.

-L83 sentence in previous version had been rewrite in revised manuscript as the following "some of these miRNAs are shown to be invertebrate-specific, which could serve as specific drug targets against schistosomiasis." (Line:109-110).

In response to Reviewer #3

1. "This manuscript is well-designed and the experiments well-executed. The work builds on their previous publication that the miRNAs Bantam miRNA and miR-1989 were related to ovarian development. This study is important as miRNAs such as Bantam miRNA and miR-1989 may be related to ovarian development and egg production. The schistosome eggs are responsible for pathogenesis and transmission of schistosomiasis. Understanding the molecular mechanisms of ovary development and egg production is important for identifying potential targets for intervention. As these miRNAs are invertebrate-specific, it makes them potentially better effective targets against schistosomiasis The current study demonstrates regulatory mechanisms for Bantam and miR-1989 in ovarian development and egg production in Schistosoma. Using siRNA, they demonstrated that Bantam and miR-1989 miRNA inhibition led to the regulation of mTOR-, Wnt- and Hippo signaling pathways. By examining the function of Bantam and miR-1989, they demonstrated that Schistosoma Frizzled proteins (Frizzled-5/7/9) were targeted by Bantam and miR-1989.

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One concern is that as one reads the results without putting some methods such as below the name of the inhibitor or the method used to inhibit, the reader has to go to the methods to understand how the result was obtained."

Author response: In the revised manuscript, we tried to include the related methods in the results. For examples, we included "using electroporation" in line 130-131;"by microscopy" in line 139; "by confocal microscopy" line 324 and others.

2. "See attached word file for minor corrections.
Line103; identify the inhibitor
Line 107 identify the control inhibitor
Line 216; rewrite in ovarian part"

Author response:
-We revised these parts accordingly.
-We rewrite these parts as the followings: "RT-qPCR analysis indicated that siRNA-476 treated worms resulted in most significant reduction of *SjRho* at the transcription level"(line 360-361) and "we observed significantly morphological alterations in ovarian architecture in the treated females with siRNA-476 that include the dilated oocyte and crack-like appearances while these changes were not observed in control siRNA-treated worms"(line 363-365).