Peer Review File
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Review Comments-reviewer A

The paper titled "Up-regulation of PUM1 by miR-218-5p promotes colorectal tumor-initiating cell properties and tumorigenesis by regulating the PI3K/AKT axis" is interesting. The result of this work points to the critical function of the miR-218-5p/PUM1/PI3K/AKT regulatory circuit in regulating T-ICs characteristics and thus suggests possible therapeutic targets for CRC. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) What are the roles of miR-218-5p/PUM1/PI3K/AKT regulatory circuit in the metastasis and molecular-targeted drug resistance of CRC? Please try to clarify the potential molecular mechanism.
   Response: This is indeed a good advice and we have added it in the discussion.

2) How does the gradual change in the molecular characteristics of CRC in the intestine affect the T-ICs in the colon and rectum? It is suggested to add relevant contents.
   Response: This is indeed a good advice and we have added it in the discussion.

3) In the supplementary results of this study, the proliferation results of miR-218-5p on CRC cells are suggested to provide BrdU staining results, which may make the results more reliable.
   Response: This is indeed a good advice; however, we cannot supplement the relevant experiments due to our experimental conditions. We have described this deficiency in the discussion.

4) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "A dual-targeted molecular therapy of PP242 and cetuximab plays an anti-tumor effect through EGFR downstream signaling pathways in colorectal cancer, PMID: 34532116", "PUM1 is upregulated by DNA methylation to suppress antitumor immunity and results in poor prognosis in pancreatic cancer, PMID: 35116535". It is recommended to quote the articles.

Response: This is indeed a good advice; We have added this in the introduction.

5) There are many genes that regulate the CRC. Why did the author choose PUM1 for research? Please describe the reason.

Response: This is indeed a good advice; We have described this in the introduction.

"PUM1, a sequence-specific RNA binding protein, participates in quite a few physiological events, for instance, the cell cycle, cell renewal, and DNA repair (9,10). (11-15). Non-small-cell lung

carcinoma (NSCLC), lymphocyte leukemia, Ovarian cancer, and other malignancies have all been shown to involve PUM1 as an oncogene (16-18). In our earlier research, we also discovered that colon cancer cells with acquired resistance to cetuximab overexpress PUM1(19). The findings from this work show that PUM1 exists as a novel biomarker for liver T-ICs and is thus a possible target for CRC therapy. In T-ICs, PUM1 is elevated and has a crucial role in colorectal cells' capacity for tumorogenicity, malignant proliferation, self-renewal, and chemoresistance."

6) What are the potential relationships between miR-218-5p/PUM1, epithelial-mesenchymal transition and cancer stem cells? How interaction of these processes may affect CRC progression, chemoresistance and ultimately recurrence? It is recommended to add relevant content.
Response: This is indeed a good advice and we have added it in the discussion.

7) There are many detection methods for cell proliferation, migration, and invasion. If multiple methods are used, the results may be more reliable. It is suggested to add test results of other methods.
Response: This is indeed a good advice; however, we cannot supplement the relevant experiments due to our experimental conditions. We have described this deficiency in the discussion.

8) What are the relevant characteristics of the tumor microenvironment of CRC? What is the correlation between miR-218-5p/PUM1 and the tumor microenvironment? What are the possible goals of future drug development? It is recommended to add relevant content to the discussion.
Response: This is indeed a good advice and we have added it in the discussion.

Review Comments-reviewer B

1. ARRIVE Checklist: We cannot find the blinding information in your paper, please check and revise.

Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Materials and methods/line 120-210

Response: Thank you for your review and we have re-edited the ARRIVE Checklist.

2. Please also define AKT in Abstract.

50 Phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. Moreover, we observed
51 that miR-218-5p directly targets the PUM1 3'-untranslated region (3'- untranslated

Response: Thank you for your review and AKT is the full name.

3. We've made minor revisions to Helsinki statement in your paper, please kindly give them a confirmation.
Response: Thank you for your review and confirm no problem.

4. Consent statement should also be added to Methods section in the main text, please check.

369 approval of the Ethics Committee of Shanghai Fourth People's Hospital, and written
370 informed consent was obtained from all participants.

Response: Thank you for your review and we have added it.

5. Study on human specimens was approved by your hospital, please kindly indicate the **approval number/ID** in your paper.
Response: Thank you for your review and this study has not applied for an ethics number.

6. We've moved the sentence to here, please confirm.

129 serum were obtained. The Shanghai Fourth People's Hospital gave its permission for
130 all animal procedures. A protocol was prepared before the study without registration.

Response: Thank you for your review and confirm no problem.

7. Animal experiment was also approved by your hospital's ethics committee, please also indicate the approval number/ID.

129 serum were obtained. The Shanghai Fourth People's Hospital gave its permission for
130 all animal procedures. A protocol was prepared before the study without registration.

Response: Thank you for your review and this study has not applied for an ethics number.

8. These two statements were duplicated, please just keep one.

129 serum were obtained. The Shanghai Fourth People's Hospital gave its permission for
130 all animal procedures. A protocol was prepared before the study without registration.
131
132 ***##In vivo xenograft and patient-derived xenograft (PDX) model***
133 BALB/c nude mice (male, 4 weeks old) were purchased from Chinese Academy of
134 Sciences Slack Company (Shanghai, China). For in vivo tumor growth assay was
135 performed as previously described (20). All procedures and protocols were approved
136 by the Ethics Committee of Shanghai Fourth People's Hospital.

Response: Thank you for your review and we have re-edited it.

9. The part in red below is missing in your paper. Please kindly provide such statement in both **Methods** section and Ethical Statement of **Footnote**.
For any experiments involving animals, the authors must indicate the nature of the ethical review permissions, relevant licenses (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals by which the research was conducted.
- *Suggested wording*: *"Experiments were performed under a project license (**NO.: the license number**) granted by institutional/regional/national ethics/committee/ethics board of *******, in compliance with ******* national or institutional guidelines for the care and use of animals."*
Response: Thank you for your review and we have re-edited it

10. Ref.22 was not cited in your paper, please cite it in order in text.
Response: Thank you for your review and we have re-edited it.

11. Figure 2: It seems the "left" and "right" here is incorrect, they do not match to your figure, please check and correct them.

507 **Figure 2.** PUM1 enhances T-ICs expansion in colorectal cancer. (A) The correlation
508 between the level of PUM1 and CD24 (left) or EpCAM (right) in primary CRC cells
509 (n = 30) was determined by real-time PCR analysis. (B) Real-time PCR analysis PUM1
510 expression in sorted CD24+ (left) or EpCAM+ (right) primary CRC cells relative to

Response: Thank you for your review and we have re-edited it.

12. Figure 3 got damaged and cannot be open, please resend us the figure in JPG/TIFF format.
And there's a typo in figure 3D, please correct it before sending us the updated figure.

1*1000	2/8	5/8	0/8	0/8
5*1000	4/8	6/8	0/8	1/8
1*10000	5/8	8/8	1/8	0/8
5*10000	5/8	8/8	1/8	2/8
Tumor idcidence	16/32 (50%)	27/32 (84.4%)	2/32 (6.3%)	3/32 (9.4%)

Response: Thank you for your review and we have resent the figure in JPG and re-edited it..

13. Figure 4

a. Please define “NS” in figure legends.

Response: Thank you for your review and we have re-edited it.

b. We cannot find “#, **” in the figure, please check and revise the legends.

556 assessed using a two-tailed Student *t*-test. *P<0.05; **, P<0.01; ***, P<0.001 #, P>0.05.

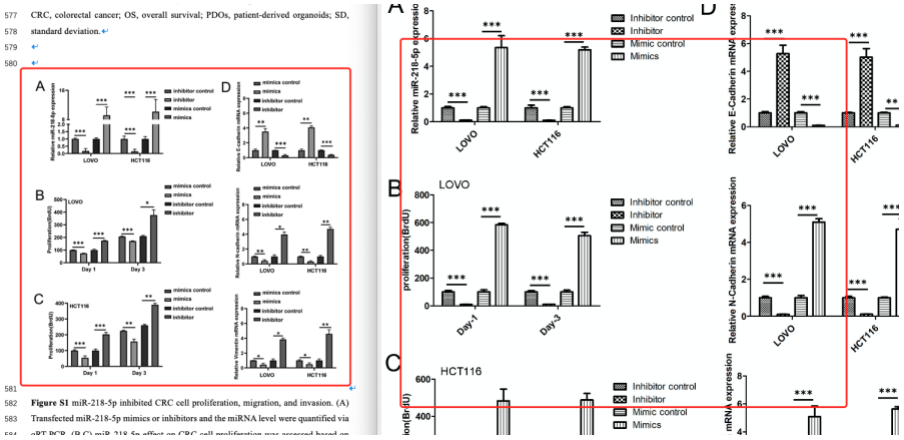
557 PUM1, pumilio homologous protein 1; 3'-UTR, 3'-untranslated region; WT, wild-type;

Response: Thank you for your review and we have re-edited it.

14. Figure 5: Please define “NS” in figure legends.

Response: Thank you for your review and we have re-edited it.

15. You’ve sent us the different Figure S1, please re-confirm which one should be the final version and re-send us the correct one as separate file.



Response: Thank you for your review and we have confirmed the final version