Dear Editor:

Thank you very much for the letter dated Dec 17, 2018, regarding our manuscript entitled "USP22 enhances intestinal cell proliferation and tissue regeneration after intestinal I/R injury" (Manuscript ID: 43267). We carefully considered the Reviewer's comments and have made revisions that are marked in red in the manuscript. We have dedicated significant effort toward revising the manuscript and have had it edited by American Journal Experts. We hope that the revised manuscript is suitable for publication in your journal. Thank you again for your reconsideration.

Point-by-point replies to Reviewers' comments are listed below.

Respectfully yours with sincere gratitude,

Xiaofeng Tian

Department of General Surgery

Second Hospital of Dalian Medical University,

Dalian, 116023, China.

E-mail: txfdl@dmu.edu.cn

Point-by-point responses to the reviewers' comments

We thank the reviewers for the constructive comments, which were very helpful for improving our manuscript. We carefully addressed all the comments from the reviewers and made additional changes throughout the manuscript. We hope that the manuscript is now acceptable. Our point-by-point responses to the reviewers' comments are listed below. Changes to the manuscript are shown in red.

Reviewer #1 **(Number ID: 02495872)**

1. English needs some polishing, as in some cases the sentences are confusing - e.g., the first sentence in the Abstract - "the mechanism of action?"

Response:

We apologize and have made revisions for those confusing sentences. Hope all the sentences now are clear in meaning and approachable to our readers.

2. First part of Introduction deals with general knowledge and importance of I/R, but there are very little references supporting the statements.

Response:

Thanks for reminding us. We have updated the references supporting the introduction and marked them in red.

3. The background on USP22 is good, but it is still not clear how the authors got into the hypothesis that it might participate in intestinal regeneration. Please improve.

Response:

Thank you for the careful and patient review of our manuscript. We have rewritten the background information (page 3, line 6-40) in the introduction section and marked them in red.

We have greatly improved the manuscript. We hope that the revised manuscript is now suitable for publication.

Reviewer #2 **(Number ID: 00503516)**

1. It is necessary to indicate the concentration of the siRNA used as well as the ratio siRNA/transfectant agent; it is also necessary to show the sequence of the control siRNA.

Response:

We are grateful for your careful and patient review of our manuscript. We have updated this information in the method section (page 5, line 17-23) and marked them in red.

2. In the result section the authors write:" Therefore, our results demonstrated that USP22 played a vital role during intestinal regeneration…" This sentence is not correct as since this point of the manuscript the authors just showed observational data about USP22 and no functional data are provided. Therefore, I suggest to modify the sentence as it follows: "Therefore, our results demonstrated that USP22 is associated to intestinal regeneration…."

Response:

Thanks for your suggestion. We have revised that part in the result section (page 7, line 10-12) and marked them in red.

3. In the result section the authors write:" Thus, our results demonstrated that USP22 can improve proliferative and regenerative activity…." Again, this sentence is not correct as the authors just showed that USP22 levels just parallel cell vitality and no

evidence of its functional role are provided. Thus, I suggest to modify the sentence as it follows: "Thus, our results demonstrated that USP22 directly correlates to the proliferative and regenerative activity…". Similarly the tile of Fig 2 legend should be modified as it follows: "USP22 correlates with…."

Response:

We have modified those sentences to the correct meaning in the result section (page 9, line 1-11) and the Fig 2 legend and have marked them in red.

4. In the result section the authors write:" … siRNA (si-USP22) dramatically decreased cell proliferation (Fig. 3B)." this sentence is not correct as fib 3B just show the correlation with the levels of Cyclin D and not with cell proliferation. I suggest to modify the sentence:" …..siRNA (si-USP22) dramatically decreased USP22 level and in parallel the levels of cyclin D and of cell vitality (Fig. 3B).".

Response:

We have revised these sentences according to your suggestion (page 10, line 6-7) and have marked them in red.

5. In figure 3 D and E the authors show the increase of G1 phase cells. Data about S and G2-M phase cells should be also reported. In the presence of a G1 block, one would expect a reduction of S and probably also G2-M phase cells. In this regard, the authors write in the discussion: "….in the G1 phase of the cell cycle and stopped them from entering S phase…" however, no data about S phase cells are shown.

Response:

Thanks for your careful and patient review. We have supplemented the data about G2 and S phase and rewritten the corresponding figure legends (Figure 3D, 3E, 4D and 4E).

6. In the result section the authors write:" ⋯ USP22 overexpression weakens I/R-induced injury and promotes cell proliferation and viability in this process" however, no data about the effects of USP22 overexpression on I/R-induced injury are shown; the authors just show the effect of in vitro cell proliferation. Thus, the words: ⋯..weakens I/R-induced injury" should be deleted.

Response:

We have revised the sentence and marked the change in red (page 12, line 10-11).

7. Lines 1-17 (Intestinal-monolayer), 32-42 (USP22-repair) and 55-61 (Cyclin D-phase) of the discussion should be condensed in the introduction and removed from the discussion. Lines 42-55 (Since-hypothesis) should be condensed as they just repeat the results obtained. In the discussion, the authors should better discuss their data in relation to previously published papers.

Response:

We have revised, condensed and moved those parts you mentioned to the introduction section. The description of the result in discussion section was condensed. We have added information and references about previously published papers to the discussion

section. Thank you very much for helping us improve the manuscript.

We have greatly improved the manuscript and submitted it to American Journal Experts for further editing. We hope that the revised manuscript is now suitable for publication.

Reviewer #3 **(Number ID:01115220)**

1. I feel that the continued use of the abbreviations I/R and H/R in the paper are unhelpful and actually reduce readability. I suggest these are written out in full throughout.

Response:

Thanks for your suggestion. We have changed the abbreviation of H/R into its full name to make the manuscript easy to understand. Considering that there are a lot of I/Rs in the manuscript and changing all of them into full name might make the manuscript too lengthy, we left the I/R as abbreviation.

2. The abstract needs to be rewritten with more information in the methods. For instance, nothing is mentioned at all about under- or over-expression of USP22 until this suddenly appears in the results section.

Response:

Thanks for your reminding. We have greatly revised the method part both in the Abstract (page 2, line 8-15) and the Methods section (page 5, line 15-22) and have marked them in red.

3. Both in the abstract and the main text, the authors have clearly shown a tendency to over-interpret the in vivo studies. These data certainly show a change in expression of USP22 which correlates with other phenomena described. This however does not imply causation. The IEC cell studies are more convincing of a direct link, but the

authors must ensure that they do not imply causation and biological linkage just from the in vivo experiments.

Response:

We agree with your opinion. The *in vivo* experiments were not able to fully imply causation. According to your suggestion, we have revised all those descriptions in the manuscript and marked them in red.

4. Some of the English can be improved. In particular the use of "in the clinic" in the Introduction. The standard idiomatic eaning of this would the ambulatory outpatient clinic rather than the intensive care unit, where these patients usually are.

Response:

Thanks for mentioning it. We have changed all the “in the clinic” into “in clinical practice” to be more accurate.

5. Overall the experiments seem appropriate, but they are curiously under-described. Several experiments are reported in the results (including but not limited to western blotting of mouse intestine, FACS with the over-expression plasmid) and a lot of important experimental details are not included. The authors need to rewrite the methods to make sure all experiments are included and must provide sufficient methodology. Important inclusions would be the type and titer of antibodies used, the nature of the expression plasmid and control plasmid. I am not sure why forward and reverse primer sequences for siRNA are given? It is only necessary to have one strand

to silence mRNA, is one actually meant to be the control sequence? What exactly does the cell counting assay measure? The inference is that this measures viable cells? In which case the authors should refrain from calling this a proliferation assay? For the immunohistochemistry the authors performed cell counting - but what size microscopic field was used?

Response:

We really appreciate your careful and patient review of our manuscript. As you said, we made a mistake on the information of siRNA sequences. According to your suggestion, we have rewritten the method section (page 5, line 3-42; page 6, line 4-24) and supplemented relevant details to make the manuscript more approachable for the readers. We hope the revised manuscript meet the requirement of publication.

6. The figure legends are rather inadequate and, in all cases, should be expanded, so that the figures can be understood in complete isolation without and recourse to the text. Additionally, the authors have included some histology: I suggest that the figure legends actually report what is being seen in each image, which indicators on the histology images if helpful. Most readers will not be pathologists by training and will need some assistance in interpreting the histology. Hence please tell the readers what they are seeing here.

Response:

We are grateful for your careful and patient review of our manuscript. We have added arrows to the histology images (Figure 1A and 1D) and rewritten all the figure legends

to better interpret the results.

7. The authors have performed some nice over and under expression studies with USP22, which obviously does support their hypothesis that USP22 directly influences cyclin D1 and proliferation. However, it is disappointing they have limited their experiments in this way so much. Surely the appropriate experiments with the over and under USP22 expression are to essentially repeat the experiments in figure 2 with the under and over expression and provide a time course for the changes in proliferation and cyclin D1 in relation to altered USP22. This is a very important point as the time course of the changes in USP22, proliferation and cyclinD1 appear to be mostly synchronous, it might be reasonable to expect the changes in USP22 to precede the others? A fuller time course would help clarify this?

Response:

We agree with your opinion. We did not observe the anticipated results in the full-time course experiment, the results only showed the synchronousness of the change in USP22, Cyclin D1 and proliferation. Your suggestion is valuable for our further study.

8. In the discussion section the authors mention the results of the FACS analysis in the USP22 overexpression system, but no data about this are included in the results section

Response:

Thanks for your suggestion. We added this data in Figure 4D and 4E (page 12), which

we ignored in the first manuscript.

9. Much of the first paragraph of the discussion is redundant and repetition of previously included information and could be removed. However, the authors do stress the importance of de novo proliferation in recovery from reperfusion injury, they do not mention other potential methods of recovery such as cell motility and wound closure? These are important in other GI tract pathologies but are they important in this system?

Response:

Thanks for your thoughtful suggestion. Indeed, there are studies in other models adopting cell motility and wound healing assay to indicate the *do novo* recovery [1, 2]. However, in our previous and present studies[3, 4], we only used classical ways. We would adopt those methods in future study and observe the migration ability of intestinal cells in our model.

10. In the methods section, the authors mention a power calculation and sample size estimate. However, they have not included the figures on this. These data should be included.

Response:

We used a software package G*Power 3.1 to priorly determine the adequate sample size (significance level α = 0.05; desired statistic power 1- β = 0.8) and the results came right after the input[5-7]. We have added information of this part in method

section of the manuscript (page 6, line 22-24).

We have greatly improved the manuscript and submitted it to American Journal Experts for further editing. We hope that the revised manuscript is now suitable for publication.

Reviewer #4 **(Number ID:00053419)**

1. Correlation of USP22 levels and changes on cell proliferation are clear while its role in intestinal damage recovery needs further studies, some of which are mentioned in the manuscript (using USP22 deficient mice, for instance).

Response:

We agree with your suggestion. We would focus on that in our further research. The forecast has been made to the discussion section (page 14, line 23-43).

2. USP22 has been shown to be associated with progression and chemoresisitance of colorectal cancer and therefore its therapeutic interest is at least dubious. Further discussion is needed.

Response:

Exactly, some latest investigations showed the correlation of USP22 with progression and chemoresisitance of colorectal cancer[8, 9], which is helpful in our model. Therefore, we discussed more in the manuscript (page 14, line 17-29). Thanks for your kind suggestion.

3. In addition to cell proliferation there might be additional effects triggered by up-regulation of USP22 that should be analyzed to define better the mechanisms involved in its postulated protective effect after I/R (PPAR gamma, ER stress....)

Response:

It is true that deeper mechanisms must be well included in the further study, about

which we released a little bit of our future research plans at the end of the discussion section (page 14, line 23-43) and we will pay attention to those aspects as well. Some inspiring studies have shown the effects of regulating USP22 in colorectal cancer and some convincing downstream molecules[9]. We would integrate that information in our further research. The rewritten part has been marked in red in our manuscript.

4. For non-specialized readers it is worth mentioning that IEC-6 cells are normal rat small intestinal epithelial cells.

Response:

The description of IEC-6 cells have been added in the revised manuscript in method section and marked in red (page 5, line 7).

5. Antibody dilutions should be indicated.

Response:

Thanks for the reminding. We have added the product code and dilution of the antibody in the section of materials and methods section (page 5, line 34-37).

6. The first paragraph on page 11 (discussion section) is a repetitive description of the results, it should be removed.

Response:

We have removed that paragraph from the Result section to make the manuscript more condensed and clearer. We really appreciate your careful and patient review of

our manuscript.

We have greatly improved the manuscript and submitted it to American Journal Experts for further editing. We hope that the revised manuscript is now suitable for publication.

References

1. Dignass, A.U., S. Tsunekawa, and D.K. Podolsky, *Fibroblast growth factors modulate intestinal epithelial cell growth and migration.* Gastroenterology, 1994. **106**(5): p. 1254-62.
2. Sumagin, R., et al., *Neutrophil interactions with epithelial-expressed ICAM-1 enhances intestinal mucosal wound healing.* Mucosal Immunol, 2016. **9**(5): p. 1151-62.
3. Zu, G., et al., *Nurr1 promotes intestinal regeneration after ischemia/reperfusion injury by inhibiting the expression of p21 (Waf1/Cip1).* J Mol Med (Berl), 2017. **95**(1): p. 83-95.
4. Liu, L., et al., *miR-381-3p knockdown improves intestinal epithelial proliferation and barrier function after intestinal ischemia/reperfusion injury by targeting nurr1.* Cell Death Dis, 2018. **9**(3): p. 411.
5. Kim, J. and B.S. Seo, *How to Calculate Sample Size and Why.* Clin Orthop Surg, 2013. **5**(3): p. 235-42.
6. Faul, F., Erdfelder, E., Buchner, A., & Lang, A.-G., *Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses.* Behavior Research Methods, 2009. **41**: p. 1149-1160.
7. Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A., *G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences.* Behavior Research Methods, 2007. **39**: p. 179.
8. Jiang, S., et al., *Ubiquitin-Specific Peptidase 22 Contributes to Colorectal Cancer Stemness and Chemoresistance via Wnt/beta-Catenin Pathway.* Cell Physiol Biochem, 2018. **46**(4): p. 1412-1422.
9. Jiang, S., et al., *MiR-30-5p suppresses cell chemoresistance and stemness in colorectal cancer through USP22/Wnt/β-catenin signaling axis.*