Dear editor,

Thank you for your response and for reviewer's comments on our manuscript entitled "ASF1B knockdown suppresses the malignant phenotype of colorectal cancer via inactivating the PI3K/AKT pathway". We have prudently considered your comments and made revisions in the revised mode. We hope that the quality of the manuscript will be certainly improved. Here is the list of our responses to reviewer's comments.

Reviewer's comments and reply:

Reviewer 1:

Review:

The study by Yu et al. investigates the role of ASF1B silencing in the progression of colorectal cancer. The authors investigated the differential expression of ASF1B in colorectal cancer and found that it is upregulated, and high expression was correlated with worse survival. The authors performed mechanistic studies (in vitro and in vivo) and showed that silencing of ASF1B reduces cell proliferation, and suppressed EMT and stemness. The authors found that AS1B may act through the PI3K/AKT pathway. The aims of this study are interesting and well explored; however, I think several points merit attention before publication at the WJG.

Comments:

Major Comments:

1. **Introduction**

a. I suggest that the second paragraph needs improvement. The authors should focus more on ASF1B (I don't think that the extent of information about FN1 and TMEM100 adds to the manuscript). I suggest that the authors provide brief information on ASF1 and its paralogs. In which molecular and biological processes are they involved? What is the role of ASF1A and especially ASF1B in cancer?

Reply: Thanks for your comment. We have deleted the information about FN1 and TMEM100. Meantime, we have added brief information on ASF1 and its paralogs, and have provided its involvement in molecular and biological processes as well as the role of ASF1B in cancers. Specific modifications are as follows:

Anti-silencing function 1 (ASF1), a conserved histone H3–H4 chaperone protein, is involved in regulation of many processes such as transcription, DNA damage repair and DNA replication [11,12]. Of note, it includes two paralogous forms: anti-silencing function 1A histone chaperone (ASF1A) and ASF1B [13]. ASF1A is primarily implicated in regulation of DNA repair and cellular senescence, while ASF1B acts as a crucial regulator of cellular proliferation and cell cycle progression [12,14]. As a subtype of ASF1, up-regulation expression of ASF1B is reported to associate with the poor prognosis of lung adenocarcinoma and breast cancer patients [15,16]. Meantime, ASF1B down-regulation is demonstrated to have the ability of anti-tumor in many cancers[19-21]. For example, ASF1B knockdown suppresses cell proliferation, and promotes cell cycle arrest and apoptosis in cervical cancer [19]. ASF1B knockdown impairs proliferation, migration and invasion of lung cancer cells [20]. Silencing of ASF1B represses growth of hepatocellular carcinoma cells, and induces cell cycle arrest [21].

b. Likewise in the same paragraph, the authors state: "what intrigues us is… database". This information should be included in the results section instead of the introduction.

Reply: Thanks for your comment. We have deleted the information mentioned above in the introduction section, and this information is included in the results section. The contents are as follows:

We firstly analyzed ASF1B expression in TCGA database, and observed up-regulation of ASF1B expression in tissues of colon adenocarcinoma and rectum adenocarcinoma in contrast to corresponding noncancerous tissues (Figure 1A, $P < 0.05$).

c. Please highlight the importance of the conduction of this study. (i.e why is the ASF1B attracting research interest?)

Reply: Thanks for your comment. We have highlighted the conduction importance of this study in the introduction section. The contents are as follows:

Anti-silencing function 1 (ASF1), a conserved histone H3–H4 chaperone protein, is involved in regulation of many processes such as transcription, DNA damage repair and DNA replication [11,12]. Of note, it includes two paralogous forms: anti-silencing function 1A histone chaperone (ASF1A) and ASF1B [13]. ASF1A is primarily implicated in regulation of DNA repair and cellular senescence, while ASF1B acts as a crucial regulator of cellular proliferation and cell cycle progression [12,14]. As a subtype of ASF1, up-regulation expression of ASF1B is reported to associate with the poor prognosis of lung adenocarcinoma and breast cancer patients [15,16]. More importantly, ASF1B down-regulation is demonstrated to have the ability of anti-tumor in many cancers[19-21]. For example, ASF1B knockdown suppresses cell proliferation, and promotes cell cycle arrest and apoptosis in cervical cancer [19]. ASF1B knockdown impairs proliferation, migration and invasion of lung cancers cells [20]. Silencing of ASF1B represses growth of hepatocellular carcinoma cells, and induces cell cycle arrest [21]. Overall, ASF1B is gaining our attention as an important player in the development of diverse cancers. Meantime, the function and mechanistic understanding of ASF1B have rarely been reported in CRC.

Our study provides new insights into the functional importance of ASF1B in CRC, and indicates that ASF1B may be a promising prognostic marker and a target for the management of CRC.

d. Could the authors add the hypothesis of this study?

Reply: Thanks for your comment. We have added the hypothesis of this study to the introduction section. The contents are as follows:

Thus, we assumed that ASF1B may regulate the PI3K/AKT pathway to affect malignant progression of CRC.

2. Material and Methods

a. Could the authors include the ethical approval registration number of this study?

Reply: Thanks for your comment. We have added the ethical approval registration number to the manuscript. Specific modifications are as follows:

The current study has got permission from the Ethics Committee of Zhebei Mingzhou Hospital (ZBMZYYLL211028).

b. Please provide information related to cancer to normal analysis on the GEPIA tool as this analysis was mentioned in the results section (Figure 1A)

Reply: Thanks for your comment. We have provided information related to cancer to normal analysis on the GEPIA tool. The specific contents are as follows:

Online website

The online website GEPIA (http://gepia.cancer-pku.cn/detail.php) was used to compare the expression of ASF1B between tumor tissues and normal tissues in CRC.

c. How did the authors decide on the sample size of the tissue specimens and the mice studies? Was a sample size calculation performed?

Reply: Thanks for your comment. We have not performed a sample size calculation. We determined the sample size of the tissue specimens and the mice studies according to previous literature and the quantity with statistical significance.

d. Please provide the status of the tissue samples (i.e fresh tissue, fresh frozen or fixed-formalin-paraffin-embedded). Was the sample collection prospective or retrospective? What were the inclusion and exclusion criteria of this sample group?

Reply: Thanks for your comment. We have provided the status of the tissue samples. The details are as follows:

Total 68 pairs of CRC tissues and adjacent normal tissues (3.0 cm away from tumor margin) were acquired from CRC patients between June 2019 and January 2021, which were transported by liquid nitrogen to the laboratory and then stored at -80°C until use.

The sample collection is prospective, and the inclusion and exclusion criteria of this sample group are as follows:

The inclusion criteria were as follows: (1) Complete general information (including gender, age, ethnicity, past history, family history, etc.); (2) Patients who underwent bidirectional endoscopy (colonoscopy performed immediately after gastroscopy). The exclusion criteria were as follows: (1) History of gastric cancer, peptic ulcer and other cancers; (2) Received antibiotics, proton pump inhibitors or glucocorticoids in the past month; (3) Patients who underwent chemotherapy, radiation therapy and other treatments for tumors; (4) Previous history of gastrointestinal surgery; (5) Presence of inflammatory bowel disease, Gardner's syndrome (a disease that affects the incidence of CRC) or familial adenoma; (6) A history of systemic diseases.

e. Please clarify if mycoplasma testing was performed in cell culture

Reply: Thanks for your comment. We have performed mycoplasma testing in cell culture. The details are as follows:

For eliminating mycoplasma contamination, all cells were routinely examined by using MycAway (Yeasen, Shanghai, China).

f. The cell transfection needs to be described in more detail: i.e: How many cells were seeded and transfected? What technique did the authors use for transfection (forward or reverse transfection?) Could the authors clarify if there was a stable or transient transfection and provide relevant methodology?

Reply: Thanks for your comment. We have described cell transfection in more detail. Specific modifications are as follows:

Then HCT116 and SW620 cells were seeded in six-well plates to adjust the cell density to 5 × 10^5/mL. When the cell confluence reached 70%-80%, above plasmids (final concentrations, 50 nM) were transiently transfected into cells through Lipofectamine 3000 (Invitrogen) for 48 h.

g. Please provide the plate size in all areas in the methods section

Reply: Thanks for your comment. We have provided the plate size in all areas in the methods section.

h. Page 8, Please add a statement regarding the compliance of the *in vivo* studies with the ARRIVE guidelines.

Reply: Thanks for your comment. We have added a statement regarding the compliance of the *in vivo* studies with the ARRIVE guidelines. Specific modifications are as follows:

Animal experiments have got permission from the Institutional Animal Care and Use Committee of Beijing Viewsolid Biotechnology Co. LTD (VS212601454), which complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as ARRIVE guidelines.

i. Page 9, line 2: What do the authors mean by "After injection for 5 weeks"? Were the injections repeated?

Reply: Thanks for your comment. We apologize for this, and we have corrected this sentence. Specific modifications are as follows:

On the 35th day after the injection, mice were anesthetized through intraperitoneally injecting sodium pentobarbital (50 mg/kg) and then euthanized through cervical dislocation.

j. In the statistical analysis information about the Kaplan Meier analysis and log-rank test need to be included in this section.

Reply: Thanks for your comment. We have revised this problem. Specific modifications are as follows:

Survival curves were plotted through the Kaplan-Meier method and differences between survival curves were assessed using the log-rank test.

k. Could the authors please clarify where the one-way ANOVA was used for multiple groups? (In the figures I could see two comparison groups control vs transfected or treated with PI3K inhibitors vs non-treated)

Reply: Thanks for your comment. We have not used the one-way ANOVA for multiple groups, and we are sorry for this error. We have deleted the one-way ANOVA in the manuscript to correct this problem.

In the clinical data, the follow-up time is more than 80 months. However, it is unreasonable to collect patient samples from 2019 to 2021. Please clarify.

Reply: Thanks for your comment. "From 2019 to 2021" is the time of sample collection not the time of follow-up, and we have added the time of follow-up to the manuscript. The contents are as follows:

Follow-up

The survival of patients was followed for 90 months through the records of reexamination or the telephone.

3. Results

a. What do the authors mean by malignant behaviour changes? Could you please clarify the outcomes (i.e proliferation, EMT)

Reply: Thanks for your comment. We have clarified the outcomes in the manuscript. The details are as follows:

Next, the impact of ASF1B on proliferation of CRC cells was probed through loss-of-function assays.

b. A table with the clinicopathological data and demographics of the patient group included is recommended

Reply: Thanks for your comment. We have added this table (table 1) to the attachment.

c. Would the authors consider performing a multivariable analysis adjusted for clinicopathological data to further assess the prognostic role of the ASF1B in CRC?

Reply: Thanks for your comment. We have performed a multivariable analysis adjusted for clinicopathological data to further assess the prognostic role of ASF1B in CRC. Moreover, we added figure 1F to assess the diagnostic role of ASF1B in CRC.

The analysis results are as follows:

We found that ASF1B expression was significantly associated with tumor node metastasis (TNM) stage, lymph node metastasis and distant metastasis (Table 1).

The expression of ASF1B was markedly higher in CRC patients at TNM stage III/IV than CRC patients at TNM stage I/II (Figure 1F, $P < 0.01$)

4. Discussion

a. I suggest that the authors should indicate the novelty of this study

Reply: Thanks for your comment. We have added the novelty of this study in the discussion section of the manuscript. The contents are as follows:

Overall, the novelty of this study included two aspects. Firstly, we demonstrated the role of ASF1B in CRC *in vitro* and *in vivo* for the first time. Secondly, we uncovered a new mechanism by which ASF1B made impact on CRC cells.

b. I think it is worth discussing further the impact of ASF1B silencing on EMT, an important cancer hallmark in metastases and the "cadherin switch".

Reply: Thanks for your comment. We have added more discussion regarding the impact of ASF1B silencing on EMT. The contents are as follows:

As an important cancer hallmark in metastases and the "cadherin switch", EMT is uncovered to initiate CRC metastasis from the primary tumor to distant sites, especially to liver and lymph nodes [36,37]. Besides, numerous studies have revealed a close association between EMT and chemo-resistance in CRC [38,39]. Thus, we inferred that ASF1B may affect chemo-resistance of CRC patients.

c. It is essential to include a paragraph mentioning the limitations of this study and how these affect the interpretation of the scientific findings.

Reply: Thanks for your comment. We have added the limitations of this study to the discussion section. The contents are as follows:

However, there were two limitations in our study. Firstly, we only explored the role of ASF1B knockdown in CRC, and the overexpression experiment of ASF1B should be performed to further validate the influence of ASF1B on CRC. Secondly, we failed to verify the ASF1B/PI3K/AKT pathway in CRC *in vivo*.

d. A paragraph mentioning the implications of this study in clinical practice and what areas the authors suggest should be explored in the context of future research is recommended.

Reply: Thanks for your comment. We hold the opinion that applying ASF1B to targeted therapy of CRC should be explored in the context of future research.

Minor Comments:

1. Please include the full text of every abbreviation on the first appearance.

   Reply: Thanks for your comment. We are sorry for our carelessness and we have modified this problem in the manuscript.

2. Please check the entire manuscript for grammar errors and typos.

   Reply: Thanks for your comment. We have asked helps from professionals and tried to correct all grammar errors and typos.

3. Page 7, line 9 What do the authors mean by "other procedures"? please rephrase for more clarity.

   Reply: Thanks for your comment. We apologize for this, and we have corrected this problem in the manuscript.

4. Could the authors rephrase line 4 in the discussion section "shows great clinical value" to avoid overstatement?

   Reply: Thanks for your comment. We have rephrased this sentence. Specific modifications are as follows:

   Emerging evidence has uncovered that ASF1B is highly expressed and shows potential clinical value for prognosis of cancer patients [15,21].

5. I suggest replacing the word "turn-out" with others such as "shown" "found"

Reply: Thanks for your comment. We have replaced the word "turn-out" with "found".

6. Figures/Legends

a. sample size should also be included in the figures or legends (i.e figure 1, figure 5)

Reply: Thank you for your comment. We have supplemented the sample size in figure 1C and figure 5D.

b. Please provide the number of biological and technical replicates in all relevant figure legends

Reply: Thanks for your comment. We are sorry to leave out this information, and we have supplemented the number of biological and technical repeats in relevant figure legends.

c. Figure 1A: Was the GEPIA web server used to generate this figure?

Reply: Thanks for your comment. Indeed, we used the GEPIA web server to generate figure 1A.

d. Figure 1D: I think there should be a separate legend describing the staining of ASF1B (nuclear and/or cytoplasmic localization of ASF1B protein).

Reply: Thank you for your comment. We have added a separate legend describing the staining of ASF1B.

e. In Figures 4B and 4D what were the comparison groups that the asterisks correspond to?

Reply: Thanks for your comment. We have added comparison groups in legends of figures 4B and 4D. The details are as follows:

sh-ASF1B#1 VS. sh-NC, sh-ASF1B+740 Y-P VS. sh-ASF1B#1

Reviewer 05776275

Comments: This manuscript showed the in vitro display that ASF1B down-regulation distinctly attenuated proliferative, migratory and invasive abilities of HCT116 and SW620 cells, and retarded EMT and stemness of HCT116 and SW620 cells. At the same time, the authors' results in vivo

indicated that ASF1B down-regulation suppressed tumor growth of xenograft mice as well as Ki67 expression. Secondly, ASFIB concept in proliferation attenuation in colorectal cancer, has been demonstrated by this manuscript This study has shown that ASF1B will impact the prognosis of CRC patients.

Reply: Thanks for your comment.

Reviewer 00505755

Comments: The study demonstrates that ASF1B may contribute in the malignant phenotype of colorectal cancer via PI3K/AKT pathway. Figure 2 may be revised to indicate the differences between sh-ASF1B#1 and sh-ASFB#2 in the legend.

Reply: Thanks for your comment. We have revised the legends of figure 2, figure 3 and figure 4A.

Revision reviewer

Comments and reply:

The authors have appropriately addressed the reviewer's comments. The quality of the manuscript has been improved, however, some points merit attention. Please find below my comments and suggestions to further improve the quality of the manuscript.

Minor comments:

1. Page2, line 21: I suggest the abstract be amended to include the new findings regarding the association of ASF1B with an advanced stage of CRC.

   Reply: Thanks for your comment. We have amended the abstract and added the new findings to the abstract. The specific contents are as follows:

   ASF1B expression was markedly increased in CRC tissues and cells, and it was inversely correlated with overall survival of CRC patients and was positively associated with the tumor node metastasis (TNM) stage of CRC patients.

2. Page 5, lines 1-3: I suggest the phrase "Our study…. The management of CRC"

be moved to the conclusion section.

Reply: Thanks for your comment. We have moved the above mentioned phrase to the conclusion section.

3. Page 10, lines 2-3: Could the authors please rephrase the sentence "Clinicopathological characteristics…. using the $\chi 2$ test."? A suggestion would be: Clinicopathological characteristics between patients who demonstrated high ASF1B expression versus those with low expression were compared using the $\chi 2$ test.

   Reply: Thanks for your comment. We have rephrased the sentence according to the above suggestion.

4. Page 10, line 6: I suggest that the title needs amendment to include that high ASF1B expression is associated with adverse clinicopathological CRC characteristics.

   Reply: Thanks for your comment. We have amended the title above mentioned. Specific modifications are as follows:

   High expression of ASF1B is observed in CRC tissues and is associated with adverse clinicopathological CRC characteristics

5. Page 10, lines 14-15. Could the authors rephrase this sentence? A suggestion would be: We further investigated the clinical significance of ASF1B in CRC and we found that ASF1B…

   Reply: Thanks for your comment. We have rephrased this sentence according to the above suggestion.

6. Page 10, lines 17-18. I suggest that the phrase "ASF1B up-regulation….. data of CRC patients" moves at the end of the paragraph. Please change the order of the figures accordingly.

   Reply: Thanks for your comment. We have put this phrase at the end of the paragraph and have changed the order of the figures accordingly.

7. Page 10, lines 22-27: I suggest rephrasing these lines. A suggestion would be: "Examination by western blot showed that ASF1B expression was increased in HT29, HCT116, LOVO, SW480 and SW620 cells compared to FHC cells ($P <$

0.001) and especially in SW620 and HCT116 cells. Therefore, the impact of ASF1B on CRC cell proliferation was investigated through loss of function assays in HCT116 and SW620 cell lines"

Reply: Thanks for your comment. We have rephrased these lines according to the above suggestion.

8. Page 13, paragraph 1:

a. The discussion regarding EMT and cadherin switch needs improvement. The authors need to discuss the in vitro findings of the observed E-cadherin downregulation and N-cadherin upregulation after ASF1B silencing within the context of the cadherin switch and EMT. The authors could discuss the following: Briefly describe the cadherin switch in EMT and how this affects the metastatic ability of CRC cells, and could mention that the silencing of ASF1B results in a reverse cadherin switch phenomenon indicating a potentially important role of ASF1B silencing in reducing the metastatic potential of CRC cells in vitro. Useful reference: «Loh, C.-Y.; Chai, J.Y.; Tang, T.F.; Wong, W.F.; Sethi, G.; Shanmugam, M.K.; Chong, P.P.; Looi, C.Y. The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. Cells 2019, 8, 1118.»

Reply: Thanks for your comment. We have revised the discussion regarding EMT and cadherin switch. The specific contents are as follows:

As an important cancer hallmark in metastases and the "cadherin switch", EMT is uncovered to initiate CRC metastasis from the primary tumor to distant sites, especially to liver and lymph nodes [36, 37]. It is reported that loss of E-cadherin can cause metastatic dissemination and activation of EMT transcription factors in cancer cells [38]. Many invasive and metastatic cancers are associated with high expression of E-cadherin, notably in prostate cancer [39], ovarian cancer [40], and glioblastoma [41], suggesting that E-cadherin facilitates metastasis in several tumors instead of inhibiting tumor progression. In addition, N-cadherin is reported to act as an indicator of ongoing EMT and N-cadherin down-regulation can cause metastatic

dissemination [42]. In our study, we found that the silencing of ASF1B results in a reverse cadherin switch phenomenon, indicating a potentially important role of ASF1B silencing in reducing the metastatic potential of CRC cells *in vitro*.

b. The information conveyed regarding EMT and chemoresistance does not seem to provide a clear message on what findings the authors discuss since chemoresistance studies have not been conducted in the current article. The discussion should focus more on the potential effect of ASF1B on the metastatic potential of CRC cells through EMT and PI3K/AKT pathway.

Reply: Thanks for your comment. We have deleted the contents regarding chemoresistance in the discussion section, and we have revised the discussion. Specific modifications are as follows:

As an important cancer hallmark in metastases and the "cadherin switch", EMT is uncovered to initiate CRC metastasis from the primary tumor to distant sites, especially to liver and lymph nodes [36, 37]. It is reported that loss of E-cadherin can cause metastatic dissemination and activation of EMT transcription factors in cancer cells [38]. Many invasive and metastatic cancers are associated with high expression of E-cadherin, notably in prostate cancer [39], ovarian cancer [40], and glioblastoma [41], suggesting that E-cadherin facilitates metastasis in several tumors instead of inhibiting tumor progression. In addition, N-cadherin is reported to act as an indicator of ongoing EMT and N-cadherin down-regulation can cause metastatic dissemination [42]. In our study, we found that the silencing of ASF1B results in a reverse cadherin switch phenomenon, indicating a potentially important role of ASF1B silencing in reducing the metastatic potential of CRC cells *in vitro*.

9. Page 13, paragraph 2: I suggest that the word "development" be deleted since the studies investigated the CRC progression.

Reply: Thanks for your comment. We have deleted the word "development".

10. Page 14, paragraph 1: I suggest the information related to the novelty of

the study be moved after the limitation paragraph.

Reply: Thanks for your comment. We have moved the information related to the novelty of the study after the limitation paragraph.

11. Page 14, paragraph 2:

a. It should be mentioned as a limitation that an a-priori sample calculation was not performed and how the authors overcome this limitation (i.e literature search).

Reply: Thanks for your comment. We have added the sample calculation to the limitation of this study. The specific modifications are as follows:

Thirdly, we failed to perform a-priori sample calculation, and we determined the sample size through literature search.

b. Additionally, I suggest that the authors could rephrase the second limitation sentence. A suggestion would be that only in vitro studies have been performed and therefore in vivo studies are recommended to strengthen the study's hypothesis.

Reply: Thanks for your comment. We have rephrased the second limitation sentence according to the above suggestion.

12. Page 14, discussion section: Thank you for replying to the comment 4d. I suggest that a short section mentioning the implications of this study in clinical practice and what areas the authors suggest should be explored in the context of future research would improve the discussion.

Reply: Thanks for your comment. We have added a short section mentioning the implications of this study in clinical practice and our future researches. The contents are as follows:

Clinical importance

ASF1B may be a potential diagnostic and prognostic biomarker for improving CRC patients' outcome. More importantly, ASF1B may be a novel target for the treatment of CRC, showing promising prospects in clinical practice. In our future studies, we plan to perform *in vivo* studies to validate the role of ASF1B in CRC. Moreover, we expect more work in investigation of the other

mechanisms of ASF1B in cancers.

13. Language improvement: Please carefully check again for grammar and typo errors.

Reply: Thanks for your comment. We have checked again for grammar and typo errors, and we have tried to correct grammar errors and typos.

14. Table 1: I suggest putting the sample size (n) on each group (high, low).

Reply: Thanks for your comment. We have added the sample size (n) to the low group and the high group.

15. Table 1: In addition to the absolute numbers the percentage would be useful to be included in each cell.

Reply: Thanks for your comment. We have provided the mean+standard deviation of ASF1B expression in each well.

16. I could not see the revised figure legends in the revised manuscript therefore I was not able to comment.

Reply: Thanks for your comment. We are sorry for that, and we have revised figure legends in the revised manuscript.

With regards,

Genhua Yu