Reply Reviewer #1:

According to the editor's strict regulation, I have carefully read and checked the article described by Lu et al. based on its scientific significance, soundness and novelty. In the present study, the authors have found FAM53B is up-regulated in pancreatic ductal adenocarcinoma tissues, and suppresses the metastasis of pancreatic ductal adenocarcinoma in vivo. According to their results, knockdown of FAM53B attenuated proliferation of pancreatic ductal adenocarcinoma cells. Additionally, immunohistochemical staining showed that FAM53B expression is associated with the polarization of M2 macrophage. Indeed, FAM53B had an ability to induce the polarization of M2 macrophage.
Although FAM53B had an undetectable effect on pancreatic ductal adenocarcinoma cell proliferation, knockdown of FAM53B suppressed the metastasis as examined by mouse model. Taken together, the authors suggest that FAM53B could contribute to the development of the novel strategy for the treatment of the patients with pancreatic ductal adenocarcinoma. Although the present study might provide certain advances in the related field, there are several concerns (see below) which should be adequately addressed before reconsideration. Major concerns Their description of Results section appeared to be quite different from the standard description (introductive part was too long). To avoid the possible confusion of the readers, the authors have to improve the description of their Results section. The aim of the present study was to confirm their hypothesis whether FAM53B could be implicated in development and/or metastasis of pancreatic ductal adenocarcinoma through the polarization of M2 macrophage.

Reply 1: Thank you for your advice.
Dear reviewers, thank you for your careful review of our research and valuable suggestions. In response to your questions and views, we have conducted further analysis and clarification to more clearly articulate the scientific contributions and observations of our research.
In our study, we did find upregulated expression of FAM53B in pancreatic ductal adenocarcinoma tissue, and we verified that knocking down FAM53B can inhibit the metastasis process of this cancer in vivo. Although we did not observe the direct effect of FAM53B on the proliferation of pancreatic ductal adenocarcinoma cells in vitro, experiments in mouse models demonstrated that FAM53B knockdown can indeed inhibit its metastasis process.
For our descriptions in the results section, we will carefully review and make necessary changes to ensure that the result descriptions are more consistent with the standard descriptions. We are well aware of the importance of the accuracy of the description for readers to understand the research, and will do our best to improve the expression of the relevant parts.
The clarification you mentioned about the purpose of the study is crucial. Our aim was indeed to investigate whether FAM53B is involved in the development and/or metastasis of pancreatic ductal adenocarcinoma by regulating the polarization of M2 macrophages. Our experimental evidence shows that FAM53B is closely related to

the polarization of M2 macrophages, and the regulation of FAM53B plays an important role in inhibiting the metastasis of pancreatic ductal adenocarcinoma. Finally, we are convinced that this study provides a useful advance in the field of pancreatic ductal adenocarcinoma treatment and may have a positive impact on treatment strategies for patients. We will pay close attention to and carefully address the issues you raise in order to improve the scientific quality and accuracy of the research.
Thank you again for your valuable comments and review work, we will continue to improve our research according to your suggestions.

FAM53B-mediated polarization of M2 macrophage had undetectable effect on proliferation as well as apoptosis of pancreatic ductal adenocarcinoma cells. The authors have to discuss why FAM53B could be involved in the metastasis but not in proliferation.

Reply 2: Thank you for your advice.
Dear reviewers, thank you for your careful review of our research and valuable suggestions. In response to your question, we have thought deeply and seriously considered the role of FAM53B in regulating the metastasis process of pancreatic ductal adenocarcinoma without showing significant effects on the proliferation and apoptosis of pancreatic cancer cells.

Our study shows that although FAM53B-mediated polarization of M2 macrophages does not have a direct effect on the proliferation and apoptosis of pancreatic ductal adenocarcarcinoma cells, this does not exclude the possibility of its involvement in the metastasis process. In exploring why FAM53B affects pancreatic cancer metastasis but not proliferation, we can explore several possible explanations:
(1) First, FAM53B may affect the ability of pancreatic ductal adenocarcarcinoma cells to metastasize through other pathways, such as affecting cell migration, invasion, and changes in the tumor microenvironment, which may play an important role in the metastasis process without directly affecting cell proliferation and apoptosis.
(2) Second, this difference may be due to complex interactions between pancreatic ductal adenocarcinoma cells and macrophages. FAM53B may mainly affect the activity and polarization of macrophages, thereby regulating their function in the tumor microenvironment, but not directly affecting the proliferation and apoptosis of pancreatic cancer cells.
(3) In addition, this difference may be related to the specificity of cell signaling pathways. As a regulatory factor, FAM53B may be more inclined to participate in specific signaling pathways or regulate specific cell functions without affecting cell proliferation and apoptosis, but it plays an important regulatory role in the metastasis process.

In our study, we aim to uncover the potential mechanism of FAM53B in regulating the polarization of M2 macrophages and the metastasis of pancreatic ductal adenocarcinoma. Although it did not show significant effects on cell proliferation and apoptosis, this does not rule out the possibility that it plays a role in the process of metastasis. We will further explore the mechanisms behind this difference to more fully understand the mechanism of action of FAM53B in pancreatic ductal adenocarcinoma metastasis. Of course, we have supplemented the discussion and analysis of this related issue in the discussion part of this manuscript according to your request, and the revised part has been marked in yellow according to the requirements of the journal. Thank you again for your suggestion.

Discussion part was composed on too many introductive descriptions. Discussion part should be described based on their present findings.

Reply 3: Thank you for your advice.
Dear reviewers, thank you for reviewing our research and for your valuable feedback. With regard to the discussion section you mentioned having too many introductory descriptions, we deeply understand your suggestion and will adjust the discussion section accordingly to focus more on the discussion based on our current research findings.
Our intent in the discussion section was to delve deeper into the effect of FAM53B on macrophage M2 polarization on pancreatic ductal adenocarcinoma metastasis, but may have indulged too much in background and introductory descriptions and failed to adequately highlight current findings. We will improve the discussion section by:
We will optimize the structure of the discourse to ensure that the discussion is tightly focused on the findings and observations of our research. We will focus more on describing the significance and possible explanations of our results, highlighting the potential mechanisms of FAM53B on macrophage polarization and pancreatic ductal adenocarcinoma metastasis. We will reduce the over-narrative of background knowledge and introductory content and focus on the findings of our research. This helps to clarify the importance of FAM53B in the regulation of macrophage M2 polarization and highlights its potential influence on pancreatic ductal adenocarcinoma metastasis. In addition, we will analyze the results of our study more systematically, and combine the results of previous studies to put forward our speculation and prospects for the future development of the research field. This will help to better integrate our research into the existing body of knowledge in the field and provide useful implications for further research.

Finally, we will streamline the discussion to highlight what is relevant to our current research, and ensure that the content is coherent and logical to better express our research findings and ideas.

Thanks again for your review and guidance, we will revise the paper according to your suggestions to improve its quality and scientific value.

Minor concerns Introduction section is too long. The authors have to focus the points and describe more compactly.

Reply 4: Thank you for your advice.
Thanks to the reviewers for their suggestions. We will refine the introduction section to focus on the core research ideas and present them more concisely. We will streamline the introduction and highlight the main points and themes of the paper to ensure readers have a clearer understanding of the key content of the research. Thanks again for your guidance, we will revise as soon as possible to improve the quality of the paper.
We have made appropriate deletion of the content of the introduction to the frontiers of this research, see the yellow font and modification marks, thank you.

In Materials and methods section: The authors have to describe the sources of the primary antibodies used for WB and ICH. All of the figure legends were poorly described. More experimental information should be incorporated.

Reply 5: Thank you for your advice.
Dear reviewer, thank you very much for your review of our research and valuable suggestions. You mentioned that the materials and methods section requires more experimental data, especially the source of primary antibody, as well as detailed descriptions of Western Blot (WB) and immunohistochemical staining (ICH). We attach great importance to your suggestions and will supplement and modify the relevant parts in detail to ensure the clarity and transparency of the experimental method.
For the description of the source of the primary antibody, we will clearly indicate the source, manufacturer or supplier of each used primary antibody in the paper, and explain the target protein of the antibody, clone model and other detailed information. This will help the reader to fully understand the source and reliability of the experimental material.
For the experimental details of WB and ICH, we will further add information on the experimental procedures, reagents used, operating conditions, antibody dilution, and signal detection methods. We will describe in detail the standardized operation procedures of the experiment, including protein extraction, electrophoresis conditions, membrane transfer conditions, dyeing conditions, etc., so that readers can replicate and verify our experimental results. At the same time, we further supplement the relevant content description content of each legend.

We are committed to providing clear and detailed descriptions of experimental methods to ensure repeatability of experiments and reliability of results. At the same time, we also thank you for your guidance, and these revisions will further improve the quality and scientific value of our papers.
Thanks again for your review and guidance. We will revise the paper according to your suggestions as soon as possible.

The efficiency of FAM53B knockdown in BXPC-3 and PANC-1 cells should be validated (RT-PCR and/or WB). Although the authors described that “This result highlights the importance of cell interactions for tumor development and provides insights into the underlying mechanisms of the pancreatic ductal adenocarcinoma microenvironment” based on the results shown in Figure 3, there was no direct evidence supporting their conclusion. CRISPR/Cas9-mediated gene silencing should be referred to as “knockout” not as “knockdown”.

Reply 6: Thank you for your advice.
Dear reviewer, thank you very much for your careful review and valuable suggestions on our paper. In response to the question you mentioned, we will verify the knockout efficiency of FAM53B in BXPC-3 and PANC-1 cells, in particular by RT-PCR and/or Western Blot (WB) assay. We understand the importance of ensuring the reliability and accuracy of your experimental results, and we will do our best to supplement and validate the results of this part of the experiment.
Regarding the insufficient supporting evidence between the results described in the paper and the conclusions, we will strengthen the interpretation of the results and support our conclusions. We will express our experimental results more clearly to ensure the scientific reliability and logical consistency of the conclusions of the paper.
At the same time, your comment that CRISPR/ Cas9-mediated gene silencing should be called "knocking down" rather than "knocking down" has also been taken seriously by us. We will correct the description in the paper to ensure that the correct terminology is used to describe the operation and effects in the gene editing experiment to maintain accuracy and normativity.
We are honored to receive your professional guidance, your comments are of great significance to our efforts to improve the quality and scientific value of our papers. We will revise and improve the paper based on your suggestions to ensure that the final content presented to the reader is more accurate and clear.

Figure 3 Supplementary content in the results section：
We then analyzed the abundance of M0 macrophages (CD68), M1-type macrophages (CD86) and M2-type macrophages (CD206) in pancreatic cancer tissues by immunofluorescence technique, and found that the number of M2-type macrophages in pancreatic cancer tissues was significantly higher than that of M1-type macrophages ($P<0.01$)(Figure 3A-B). In addition, we found that TNFSF9 was mainly

expressed on immune cells by fluorescence colocalization analysis ($P<0.001$)(Figure 3C). Moreover, the expression of TNFSF9 was more correlated with M2 macrophages ($r=0.722$) than M1 macrophages ($r=0.599$)(Figure 3D).

Reply Reviewer #2:

The manuscript titled “FAM53B promotes pancreatic ductal adenocarcinoma metastasis by regulating macrophage M2 polarization” attempts to demonstrate that FAM53B promotes pancreatic ductal adenocarcinoma metastasis by regulating macrophage M2 polarization. However there are several major issues that need to be clarified before the manuscript can be considered for acceptance.

Reply 1: Thank you for your advice.
Dear reviewer, thank you for your review and valuable comments on our paper. We understand that there are several major issues with the paper that need to be clarified before you can consider accepting the manuscript.

The aim of this study was to elucidate the regulatory role of FAM53B on the metastasis of pancreatic ductal adenocarcinoma, especially through the regulation of macrophage M2 polarization. We will focus on clarifying the following key issues to enhance the scientific and credibility of the paper: First, we will elaborate the experimental design and methods of the study in more detail to ensure the rigor and repeatability of the experimental process. In particular, regarding the exact mechanism of FAM53B in regulating the polarization process of M2 macrophages, we will add more experimental data and analysis to support our argument. Secondly, we will further emphasize the reliability of experimental results and provide clearer data presentation to ensure that our conclusions are based on sufficient experimental evidence. This includes a more comprehensive and accurate interpretation of experimental results on FAM53B's regulation of macrophage M2 polarization and its effect on pancreatic ductal adenocarcinoma metastasis. Finally, we will strengthen the clear expression of the main idea of the paper to ensure that the title and abstract of the paper accurately summarize the core content of our study, namely, the effect of FAM53B on pancreatic ductal adenocarcinoma metastasis during the regulation of macrophage M2 polarization.

We deeply appreciate your valuable suggestions, and we will seriously consider and revise and improve the paper to solve the existing problems and enhance the scientific and credibility of the paper. I look forward to presenting more excellent research results under your guidance.

Although the authors state that the manuscript has undergone language editing, the manuscript still requires significant grammar, language and sentence construction editing to make it more readable and clear. As such the text is very confusing and difficult to read.

Reply 2: Thank you for your advice.
We will send the revised manuscript to Professor Wong Wing-chan of the University of Hong Kong and Professor Ni Qianqian of the National University of Singapore for

further grammar revision and proofreading. In addition, we will further send this manuscript to AJE Company in Boston, USA for the paid service of English grammar editing and polishing. Please see the attachment for the specific English language editing certificate. Thank you again for your suggestion.

The methods section significantly lack details in protocols and are confusing. For example, in section 1.1 the authors state"…blown and mixed…" This makes no sense.

Reply 3: Thank you for your advice.
The contents of 1.1 have been deleted, modified and supplemented according to your suggestions. Thanks again for your suggestions.

In the methods section 1.2 the authors state that "… cell suspension was absorbed and added…" this has no meaning.

Reply 4: Thank you for your advice.
The contents of 1.2 have been deleted and modified according to your suggestions. Thanks again for your suggestions.

In section 1.3 the authors talk about the addition of serum free media but do not mention how much.

Reply 5: Thank you for your advice.
According to your requirements, we have supplemented the dosage of serum free media and other contents in part 1.3. Thank you again for your comments.

1.3 Preparation of conditioned medium
BXPC-3 and PANC-1 cells(CL-0019, Pricella, Wuhan, China) were inoculated in 6-well plates at a density of 1×10^5. After 24 hours of culture, the old medium was aspirated, the cells were slightly washed with sterile PBS solution twice, and then the serum-free medium(10ml, A2656101, Thermo Fisher, Massachusetts, United States) was added for 48 hours.

Section 1.4 what is 800rmp/min?

Reply 6: Thank you for your advice.
This is the speed of centrifuge： 800 rmp/min . We have supplemented the contents of part 1.4 according to your requirements. Thanks again for your suggestion.

1.4 U937 cells induced differentiation

When U937 cells (CL-0022, Pricella, Wuhan, China) grew to the logarithmic growth phase, the cell suspension was transferred to a centrifuge tube, the speed of centrifuge with 800 rpm/min for 5 minutes, poured off the supernatant, added an appropriate amount of complete medium to re-suspend the cells, and counted under the microscope. Then the cell suspension containing 1×10^6 cells was transferred into a 6 cm petri dish, and phorbol 12-myristate 13-acetate (PMA), which was prepared at a concentration ratio of 100 ng/ml, was added to the dish. Shake and mix, then put into

the cell incubator for 48 hours to differentiate into macrophage-like U937 cells.

Section 1.5 details of the CRISPR system are not provided, specifically the constructs and sequence of the gRNA. No details are provided about the cell lines used and the culture method.

Reply 7: Thank you for your advice.
In accordance with your suggestions, we have added CRISPR related information in Section 1.5, especially the structure and sequence of gRNA, as well as detailed information on cell line culture methods(Section 1.2) , etc.

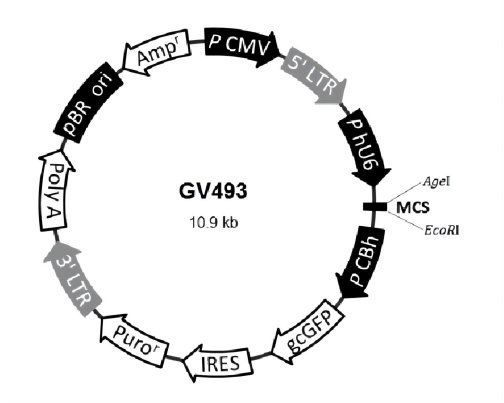
Thank you again for your suggestions.

After revised in Section 1.5:

In this study, we performed FAM53B gene knockdown using CRISPR/Cas9 technology, and constructed stable FAM53B knockdown pancreatic cancer cell lines through lentiviral infection. The virus with stable FAM53B knockdown was purchased from Shanghai Jikai Company. The vector of the virus was GV493, which

carried the expression of green fluorescent protein and the resistance to purinomycin, and the virus titer was higher than 1E+8TU.

The two shRNA sequences used in this chapter are:
sh1:5'-CTACTATGTCTTCTTTCAACT-3'
sh25 '-TGGAATACGCCTCTGACGCTT-3'.



Section 1.6 there is no mention of the macrophage model system used, what cell line?

Reply 8: Thank you for your advice.
The macrophage model was constructed using U937 cells (in Section 1.4), which we further explained in Section 1.6 according to your requirements. Thank you again for your suggestions.

After revised in Section 1.6:

The macrophage model was constructed using U937 cells, and macrophage-like phenotype U937 cells were transited to 6-well plates and transfected when the cell density grew to between 20% and 40%. Then, under the condition that PMA (propylene glycol methyl ether acetate) persisted, IL-13 and IL-4 (20 ng/mL each) were added to M0 macrophage culture medium, and the culture was stimulated for 48 h. Promote M0 macrophages (macrophages in resting state) to differentiate into M2 macrophages, thus obtaining THP-1 cell-derived M2 macrophages.

Section 1.7 what is RAPI lysate? Section 1.7 how were the exosomal vesicles collected? And how was the concentrations of the marker proteins adjusted and determined in the lysate.

Reply 9: Thank you for your advice.
At that time, considering the limited number of words in the article, we did not specifically describe this part of the content, for which we are deeply sorry.

We have added a description of RAPI lysates to your request in section 1.7. How to collect exosome vesicles? As well as an explanation of related issues such as how the concentration of labeled proteins in the lysate is adjusted and determined, thanks again for your advice.

After revised in Section 1.7 ：

The RIPA Lysis Buffer (RIPA Lysis Buffer) is a traditional fast lysis solution for cell tissue. The protein samples obtained from RIPA lysate can be used in conventional Western, IP, etc.
The PKH67 Fluorescent Cell Linker Kit is used to identify and stain exosome suspensions with high purity obtained from isolation and extraction. The pipette was used to absorb exosomes quickly and smoothly (50 μL), followed by fluid Diluent C (250 μL) after changing the gun head, and diluent C (1.5 μL) after changing the gun head again. The labeled and diluted exosomes were then completely suspended with DMEM, and the exosomes were then added to the U937 cells (DAPI staining) culture supernatant with a pipette gun. After 24 h of culture, fixation, membrane rupture, nucleation, laser scanning confocal fluorescence microscopy was used to observe whether U937 cells could take up macrophage-derived exosomes.

Section 1.8 the animal experiments lack details and make no sense. The details of the mice, age and weight rangers not indicated. Why were the cells digested? How were the animals implanted with the cancer cells?

Reply 10: Thank you for your advice.
Thank you very much for your valuable suggestions. We have re-writed that the specific details of the relevant experimental animals according to your request, including the details of the mice, age and weight, and how to grow tumor cells, etc. Thank you again for your suggestions.

After re-write in Section 1.8:

The 4-week-old female BALB/c nude mice were fed under standard pathogen-free conditions and divided into two groups of 7 mice per group. After 2 weeks of feeding, the animals can be used to build models. Firstly, the transfected PANC-1 cells were digested, centrifuged, and re-suspended to prepare a 5×10/100μl cell suspension for use. Next, the nude mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (50mg/kg), placed in the right lying position, and disinfected with 75% alcohol. A longitudinal incision is made on the left side of the abdomen, approximately 1.0 cm in length. Expose the spleen and gently pull it out of the abdominal cavity. A 1ml syringe was inserted into the lower pole of the spleen about 1.0cm and the tumor cell suspension was injected slowly. After injection, quickly pull

out the syringe, press the eye of the needle with a cotton ball of iodophenol for 2 minutes, and close the abdomen. Postoperative routine feeding. After 4 weeks of culture, the nude mice were sacrificed for cervical dislocation, and the splenic tumor formation, liver metastasis and other metastases were observed anatomically. Splenic graft tumors and liver metastases were collected and the results were observed by hematoxylin-eosin staining. The experiment was divided into two groups: sh-NC PANC-1 and sh-FAM53B PANC-1.

Section 1.10 the usage of future tense is confusing.

Reply 11: Thank you for your advice.
Thank you for your reminding. We have proofread and revised the English grammar situation and other problems in Section 1.10 again, and adjusted the tenses. Thank you for your suggestion.

No mention is the methods about how tissues were collected and processed for ICH and H&E staining. No quantification methods provided. Complete lack of details.

Reply 12: Thank you for your advice.
We added a new Section 1.9 to specifically describe the related problems you mentioned, including specific descriptions of ICH and H&E staining details. Thanks again for your suggestions.

A revised supplement:

1.9 ICH and H&E staining
ICH: The slices were washed in PBS solution 3 times for 5 minutes each time. After finishing, the slices were taken out and placed in a dark box, the excess PBS around the tissue was absorbed by filter paper, and appropriate amount of anti-rabbit and anti-mouse fluorescent secondary antibody was added, and then incubated in a 37℃ incubator for 30 minutes (the subsequent steps were all operated in a dark room). Remove the slices, put them on a slide rack and wash them in PBS solution 3 times for 5 minutes each time. After the slices were taken out and placed in a dark box, appropriate amount of DAPI dye was added and stained for 10 minutes. After the slices were rinsed in PBS solution for 3 times, they were placed in the dark box and 10 μl anti-fluorescence quench agent was added. The number of stained positive cells under 200 times of visual field was counted by fluorescence microscope, and 5 visual fields were randomly counted. These data represent the average of the results obtained by the two scorers.
H&E staining: The slide frame was soaked in hematoxylin dyeing solution for 5

minutes, washed with running water, then continued to soak in 1% hydrochloric alcohol for 3 seconds, washed again with running water, the slide frame was dyed in eosin dye for 1 minute, and washed with running water. The slide holder was placed in 50% ethanol for 5 minutes, 75% ethanol for 5 minutes, 85% ethanol for 5 minutes, 95% ethanol for 5 minutes, 100% ethanol for 5 minutes, and finally placed in two cylinders of xylene solution for 15 minutes each.

Methods are incomplete, no details about the colony formation assay, FACS analysis or the proliferation assay is provided.

Reply 13: Thank you for your advice.
We added a new Section 1.10 to specifically describe the related problems you mentioned, including specific descriptions of colony formation assay and FACS analysis details. Thanks again for your suggestions.

A revised supplement:

1.10 Colony formation and FACS analysis
Colony formation assay: PDAC cells were separately packed into culture dishes containing medium. FAM53B overexpression vector was transfected into one group and the other group was used as a blank control. After culture for a period of time, the cells were fixed with the AGAR flower tumor medium, and the number and size of the colonies formed were recorded.
Flow cytometry (FACS) analysis: Macrophages were treated to differentiate into M2 type and divided into different groups (FAM53B overexpression group and control group). Fluorescent markers are used to label surface markers or cytokines, and the proportions of different cell subpopulations and the expression levels of specific markers are detected and analyzed by FACS.

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RE-REVIEW REPORT OF REVISED MANUSCRIPT

Name of journal: *World Journal of Gastrointestinal Oncology*

Manuscript NO: 89997

Title: FAM53B promotes pancreatic ductal adenocarcinoma metastasis by regulating macrophage M2 polarization

Provenance and peer review: Unsolicited Manuscript; Externally peer reviewed

Peer-review model: Single blind

Reviewer's code: 03060131
Position: Peer Reviewer
Academic degree:
Professional title:
Reviewer's Country/Territory: Reviewer_Country
Author's Country/Territory: China

Manuscript submission date: 2023-11-20

Reviewer chosen by: Xin-Liang Qu

Reviewer accepted review: 2024-01-22 03:13

Reviewer performed review: 2024-01-24 02:45

Review time: 1 Day and 23 Hours

Scientific quality	[ ] Grade A: Excellent [ ] Grade B: Very good [ ] Grade C: Good [Y] Grade D: Fair [ ] Grade E: Do not publish
Language quality	[ ] Grade A: Priority publishing [ ] Grade B: Minor language polishing [Y] Grade C: A great deal of language polishing [ ] Grade D: Rejection
Conclusion	[ ] Accept (High priority) [ ] Accept (General priority) [Y] Minor revision [ ] Major revision [ ] Rejection
Peer-reviewer	Peer-Review: [Y] Anonymous [ ] Onymous

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statements	Conflicts-of-Interest: [ ] Yes [Y] No

SPECIFIC COMMENTS TO AUTHORS

Based on the answering part of the revised version of the article, I have carefully read and checked the revised version of the article described by Pei et al. Although the revised article might be partially improved, there are several concerns (see below) which have to be adequately addressed before publication.

Major concerns: Almost all the Results sections contained the description of results plus the related discussion. In standard article, the Results section should be composed of the description of the results not of the related discussion.

Reply 1. Thank you for your advice.

I have deleted and modified the content of the results according to your suggestions, deleted most of the discussion on the experimental research results, and modified it into an explanation of the objective content of the research and test results. I have marked the specific changes in yellow font in the manuscript, or see the screenshot below in the modification mark. Thank you again for your professional revision suggestions.

2. Result

2.1 FAM53B expression is high in pancreatic ductal adenocarcinoma

In this study, 30 pancreatic cancer tissue samples were collected, and further immunohistochemical staining experiments verified that the expression of FAM53B in pancreatic cancer was indeed significantly higher than that in adjacent non-tumor tissues ($P<0.001$) (Figure 1A-C). In addition, the study continued to culture pancreatic cancer cells (ASPC-1, PANC-1, BXPC-3, and COLO357) and normal pancreatic cells (HPDE6-C7) and verified FAM53B expression in these cells by qRT-PCR assay and protein immunoblotting assay. We found that at the protein level, the expression of FAM53B in ASPC-1, PANC-1, and BXPC-3 was significantly higher than that in HPDE6-C7 ($P<0.05$) (Figure 1D). At the mRNA level, FAM53B expression was significantly higher in ASPC-1, PANC-1, and BXPC-3 than in HPDE6-C7 ($P<0.05$), while

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FAM53B expression was not different from HPDE6-C7 in COLO357 (Figure 1E-F).

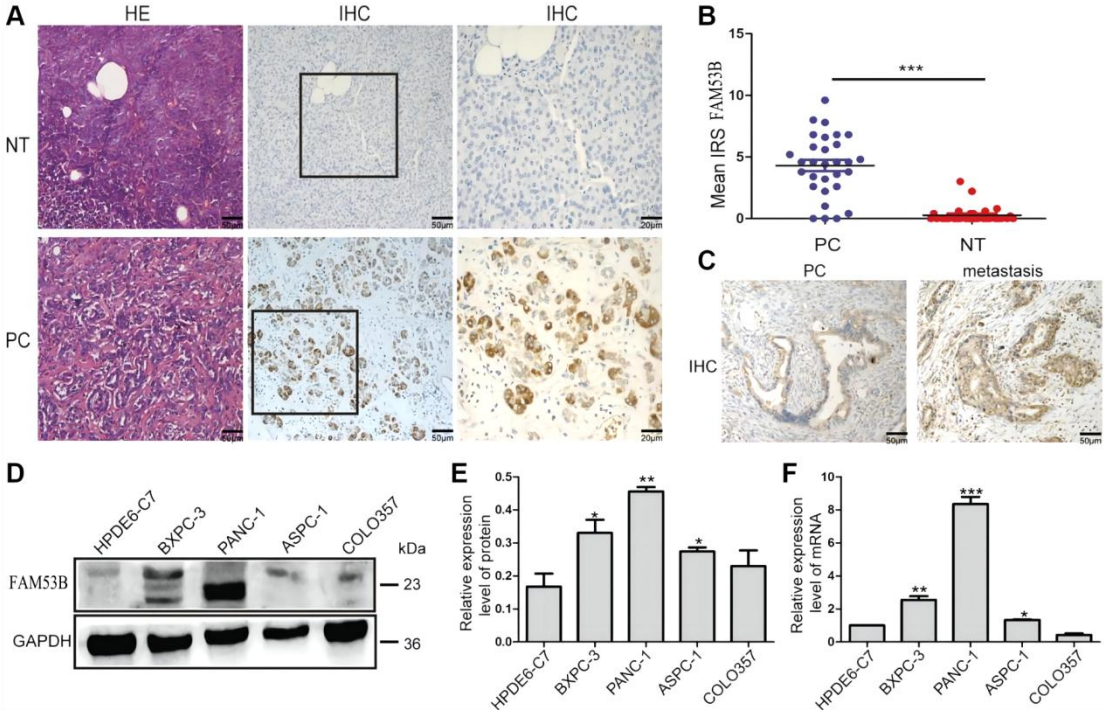

Figure 1. FAM53B expression in pancreatic ductal adenocarcinoma tissue

(A) HE staining of pancreatic ductal adenocarcinoma and para-carcinoma tissue (N=30). (B、C) Immunohistochemical staining for immune response score (NT for paracancer non-tumor tissue, PC for pancreatic ductal adenocarcinoma tissue). *P <0.05, **P <0.01, ***P <0.001. (D、E) Western blot analysis. (F) QPCR analysis

2.2 FAM53B knockout inhibited pancreatic ductal adenocarcinoma cells

The effects of sh-NC, sh-FAM53B-1 TAMs, and sh-FAM53B-2 TAMs on the proliferation of pancreatic cancer cells were studied by CCK8 and single-cell cloning experiments. These two types of TAMs, sh-FAM53B-1 and sh-FAM53B-2, did not change the growth of BXPC-3 and PANC-1 cells compared to when they were co-cultured with sh-NC TAMs (Figure 2A–F). In addition, our study found that macrophages with FAM53B knockdown promoted apoptosis of BXPC-3 and PANC-1 compared with the sh-NCTAMs group (P<0.05) (Figure 2G-J).

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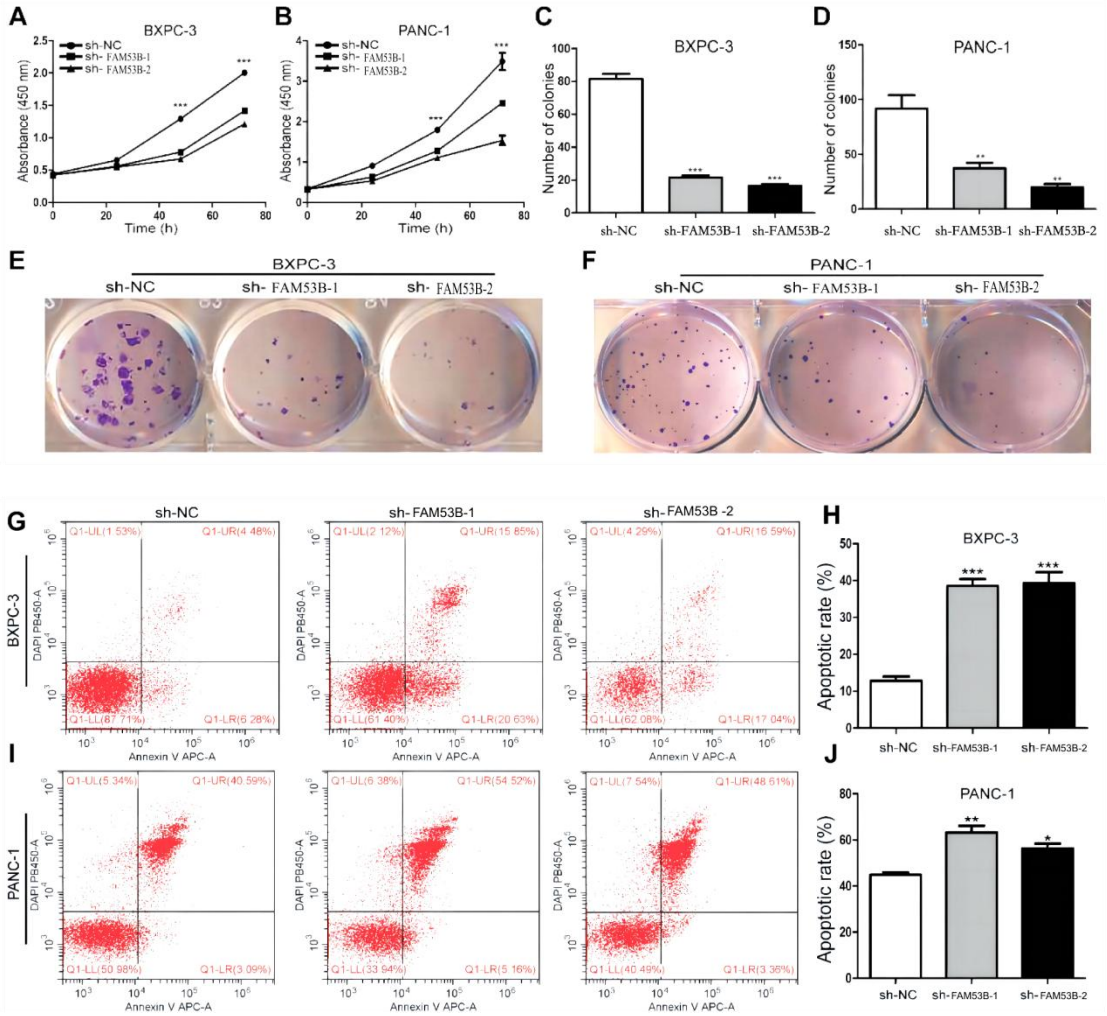

Figure 2. FAM53B knockout inhibited the proliferation of pancreatic ductal adenocarcinoma cells

(A, B) Cell counting kit-8. (C – F)Colony formation. (G–J) Flow cytometry. *P < 0.05, **P < 0.01, and ***P < 0.001.

2.3 FAM53B expression associated to polarization of M2

Our study analyzed the abundance of M0 macrophages (CD68), M1-type macrophages (CD86) and M2-type macrophages (CD206) in pancreatic cancer tissues by immunofluorescence technique, and found that the number of M2-type macrophages in pancreatic cancer tissues was significantly higher than that of M1-type macrophages (P<0.01)(Figure 3A-B). In addition, we found that TNFSF9 was mainly expressed on immune cells by fluorescence colocalization analysis (P<0.001)(Figure 3C). Moreover, the expression of TNFSF9 was more correlated with M2 macrophages (r=0.722) than M1 macrophages (r=0.599)(Figure 3D).

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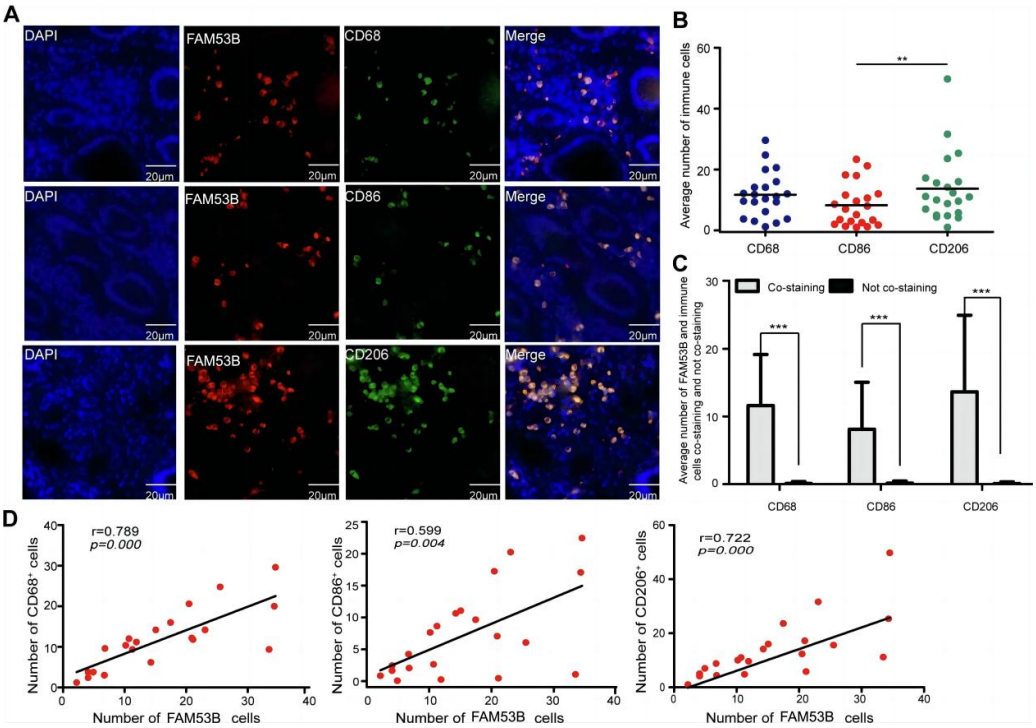

Figure 3. FAM53B expression associated to polarization of M2

(A-C) Tissue immunohistochemical test. (D) FAM53B(+) associated to M2. **P <0.01， ***P <0.001.

2.4 FAM53B induces the polarization of M2

Our study used a cellular immunofluorescence assay to compare the expression of M1 marker (CD86) and M2 marker (CD206) in knockdown FAM53B (sh-FAM53B-1 TAMs and sh-FAM53B-2 TAMs) and transfected empty vector (sh-NC TAMs) TAMs. The results showed that both BXPC-3 cell supernatant and PANC-1 cell supernatant induced TAMs, compared with the sh-NC group. The expression of the M2-type macrophage marker in sh-FAM53B-1 TAMs and sh-FAM53B-2 TAMs was significantly decreased (P<0.05), and the expression of the M1-type macrophage marker was not significantly different from that in the sh-NC group (Figure 4A-E). Then we used qRT-PCR to detect mRNA expression levels of M1 markers (IL-8 and TNF-α) and M2 markers (IL-10 and TGF-β) in sh-FAM53B-1TAM, sh-FAM53B-2 TAMs, and sh-NC TAMs. It was found that compared with sh-NC TAMs, the expression of M1 markers in sh-FAM53B-1 TAMs and sh-FAM53B-2 TAMs was increased, and the expression of M2 markers was significantly decreased (all P values <0.05) (Figure 4F-G).

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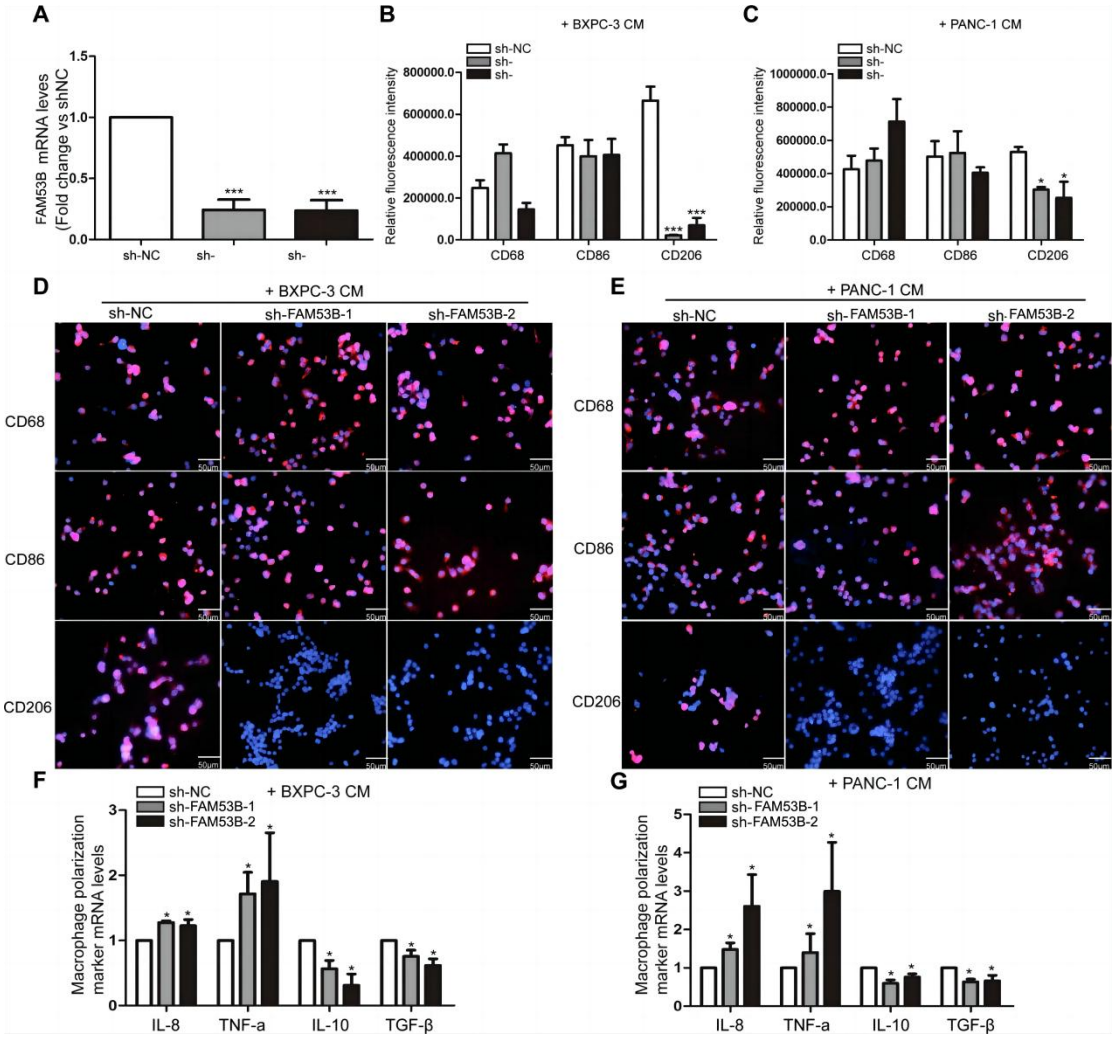

Figure 4. FAM53B induces the polarization of M2

(A-D) TAMs induced. (E-F) Expression of M1 macrophage markers. *P <0.05, ***P <0.001.

2.5 M2 polarized macrophages have no effect on the proliferation

The effects of sh-NC, sh-FAM53B-1 TAMs, and sh-FAM53B-2 TAMs on the proliferation of pancreatic cancer cells were studied by CCK8 and single-cell cloning experiments. These two types of TAMs, sh-FAM53B-1 and sh-FAM53B-2, did not change the growth of BXPC-3 and PANC-1 cells compared to when they were co-cultured with sh-NC TAMs (Figure 5A–F). We continued to compare the effects of FAM53B knockdown macrophages on pancreatic cancer cell apoptosis and found that, compared with the sh-NC-TAMs group, FAM53B knockdown macrophages promoted the apoptosis of BXPC-3 and PANC-1 (P<0.05) (Figure 5G-H).

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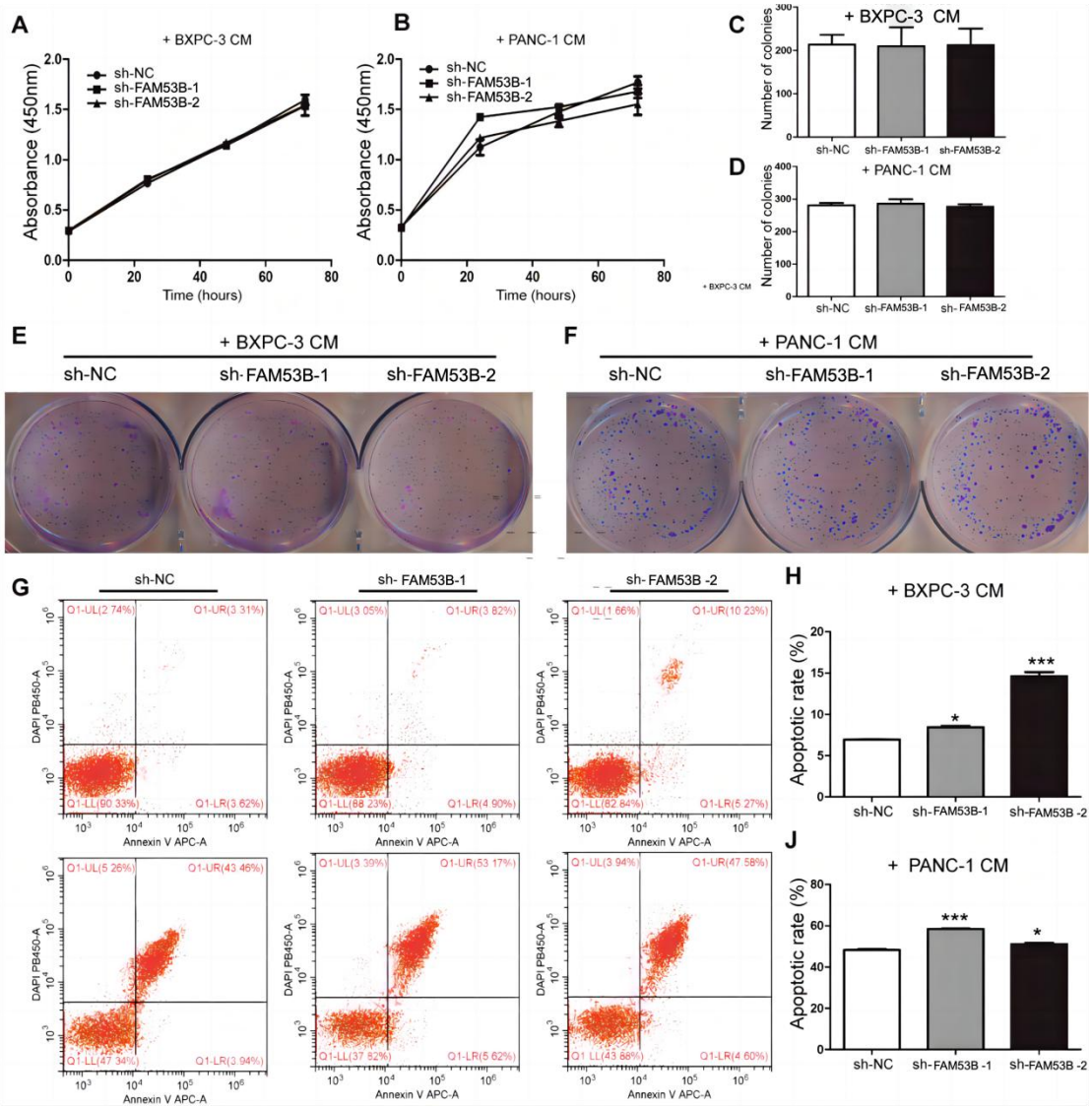

Figure 5. M2 polarized macrophages have no effect on the proliferation

(A-B) CCK8 experiment. (C-F) Single cell cloning experiment. (G-J) Flow cytometry experiment.

2.6 M2 polarized macrophages promote the development and invasion of pancreatic ductal adenocarcinoma cells

We used Transwell and matrix gel experiments to learn more about how down-regulated FAM53B macrophages affect the migration and invasion of pancreatic cancer cells. We found that sh-FAM53B-1TAMs and sh-FAM53B-2 TAMs significantly reduced the migration and invasion capacity of BXPC-3 and PANC-1 cells compared to sh-NC TAMs ($P<0.001$) (Figure 6A-D). In addition, we used western blot to detect the expression of EMT-associated proteins (N-cadherin, vimentin, and E-cadherin) after the intervention of Fam53B-knocked macrophages in pancreatic cancer cells. Sh-FAM53B-1 TAMs and sh-FAM53B-2 TAMs down-regulated the expression of

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N-cadherin and vimentin in BXPC-3 and PANC-1, and compared with the sh-NC TAMs group, the expression of N-cadherin and vimentin in BXPC-3 and PANC-1 decreased significantly. The expression of E-cadherin was significantly up-regulated (P<0.05) (Figure 6E-H).

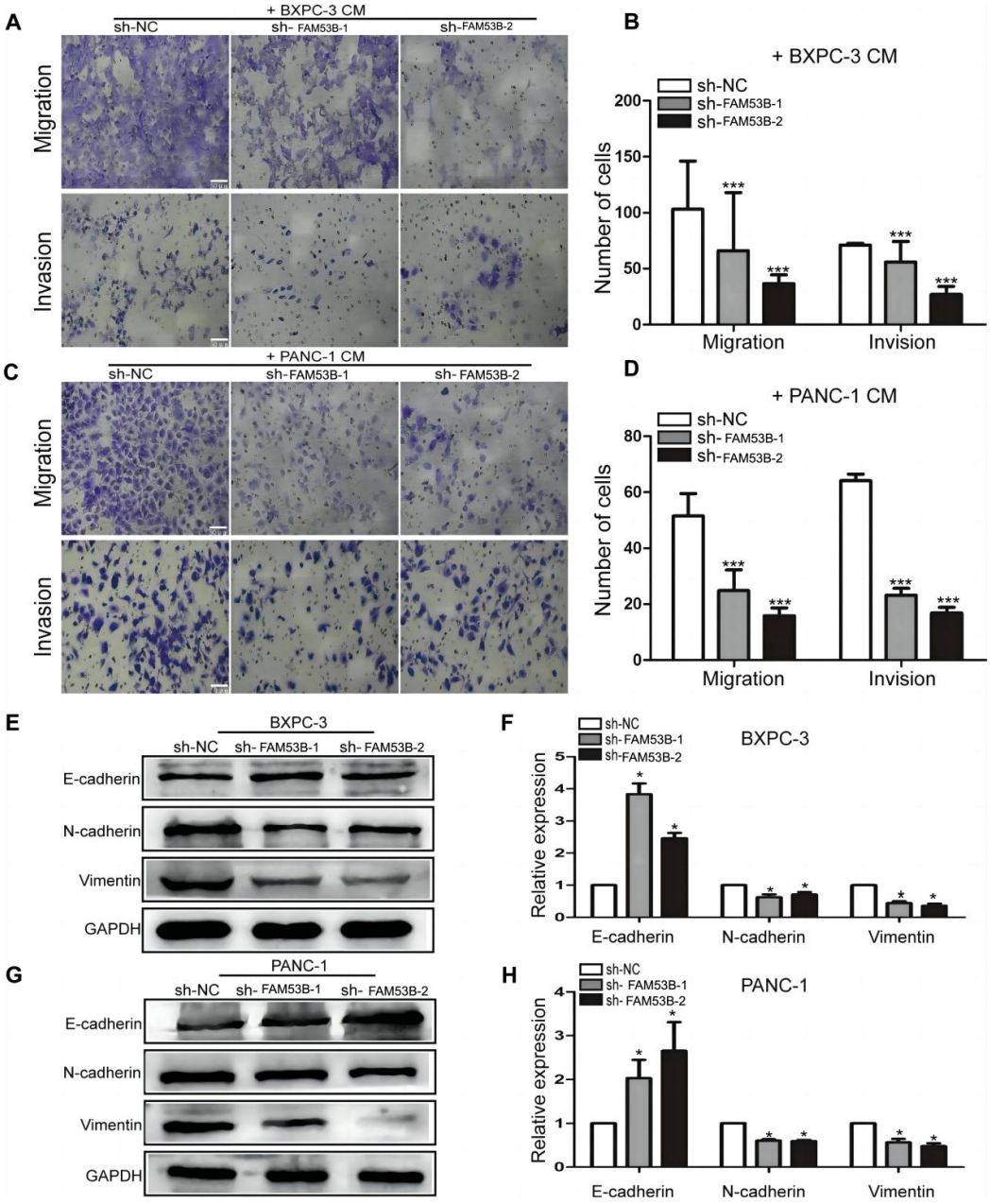

Figure 6. M2 polarized macrophages promote the development and invasion of pancreatic ductal adenocarcinoma cells

(A-D) Transwell experiment. (E-H) WB experiment. *P <0.05.

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2.7 FAM53B induces M2 polarization by activating the Src/FAK/p-Akt/IL-1 β signaling pathway

Our study further investigated the role of the Src/FAK/p-Akt/IL-1β signaling pathway in FAM53B knockdown macrophages. We found that after knocking down FAM53B in BXPC-3-induced TAMs, the expressions of Src,FAK, p-AKT, and IL-1β in sh-FAM53B-1TAMs and sh-FAM53B-2 TAMs were significantly lower than those in sh-NC TAMs. However, the expression of AKT was increased ($P<0.05$) (Figure 7A-B), while the expression of P-ERK was not significantly changed. Similarly, after knocking down FAM53B in PANC-1-induced TAMs, the expressions of Src, FAK, P-Akt, and IL-1β in sh-FAM53B-1 TAMs and sh-FAM53B-2TAMS were significantly lower than those in the sh-NC TAMs group ($P<0.05$). P-erk expression was also decreased, while AKT expression was increased ($P<0.05$) (Figure 7C-D).

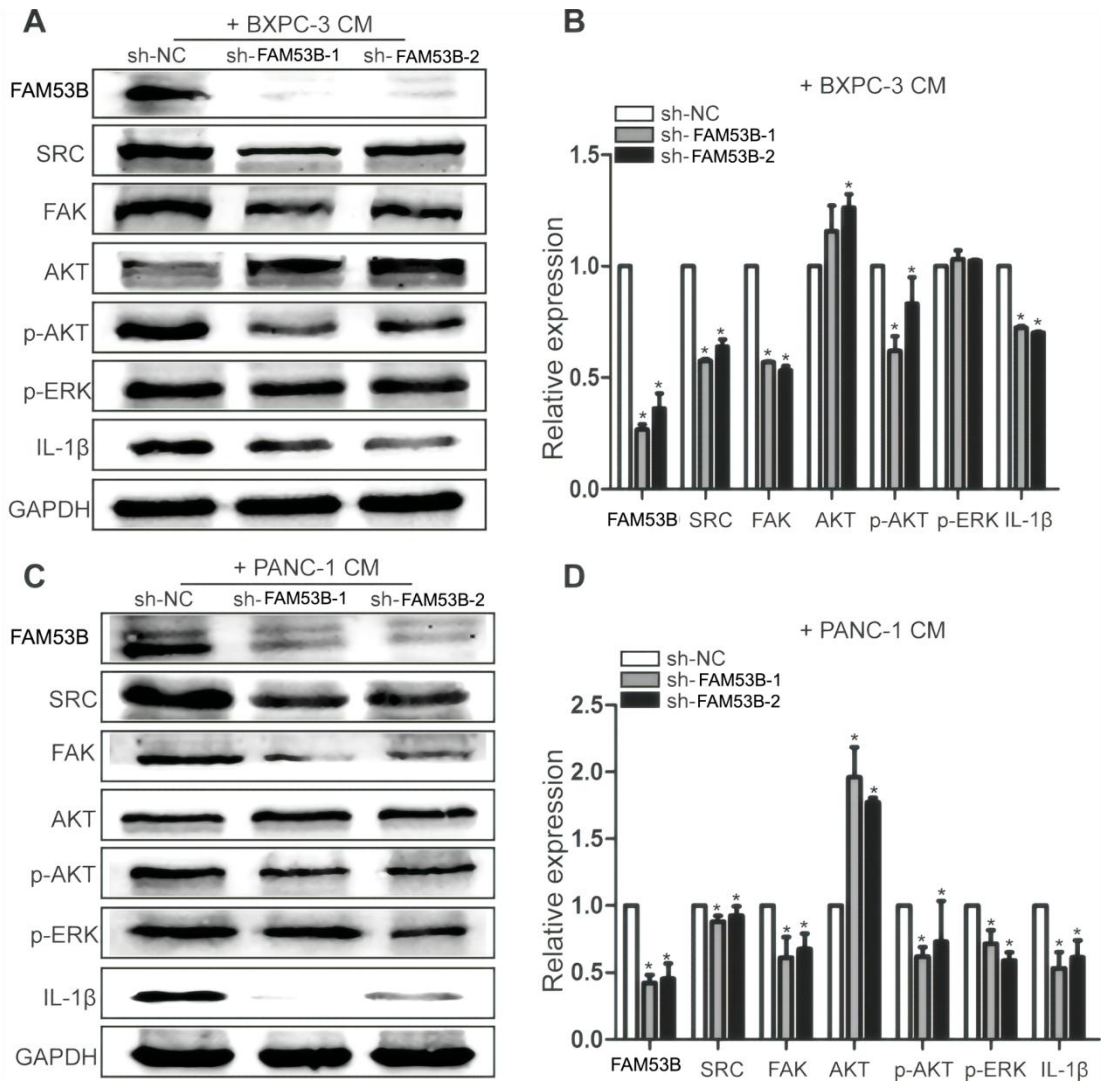

Figure 7. FAM53B induces M2 polarization by activating the Src/FAK/p-Akt/IL-1β signaling

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pathway

(A-B) FAM53B knockout in induced pancreatic ductal adenocarcinoma TAMs. (C-D) FAM53B knockout in PANC-1- induced pancreatic ductal adenocarcinoma TAMs.

2.8 FAM53B induces the metastasis of pancreatic ductal adenocarcinoma

To create a metastasis model, nude mice had PANC-1 cells knocked down by FAM53B injected into their spleens. We discovered that all of the naked mice in the sh-NC group had clear liver metastasis—a metastasis rate of 100%. This showed up as grayish-yellow spots of different sizes on the liver surface that were spread out and partially fused together into chunks. No normal spleen tissue was seen, but all were tumor tissue (Figure 8A). Liver metastasis occurred in 2 out of 7 nude mice in the Sh-FAM53B gene knockout group, and the metastasis rate was 28.6%, which was significantly lower than that in the sh-NC group. Normal spleen tissue was still visible. We sectioned liver and spleen tissue and performed HE staining to confirm the results (Figure 8B).

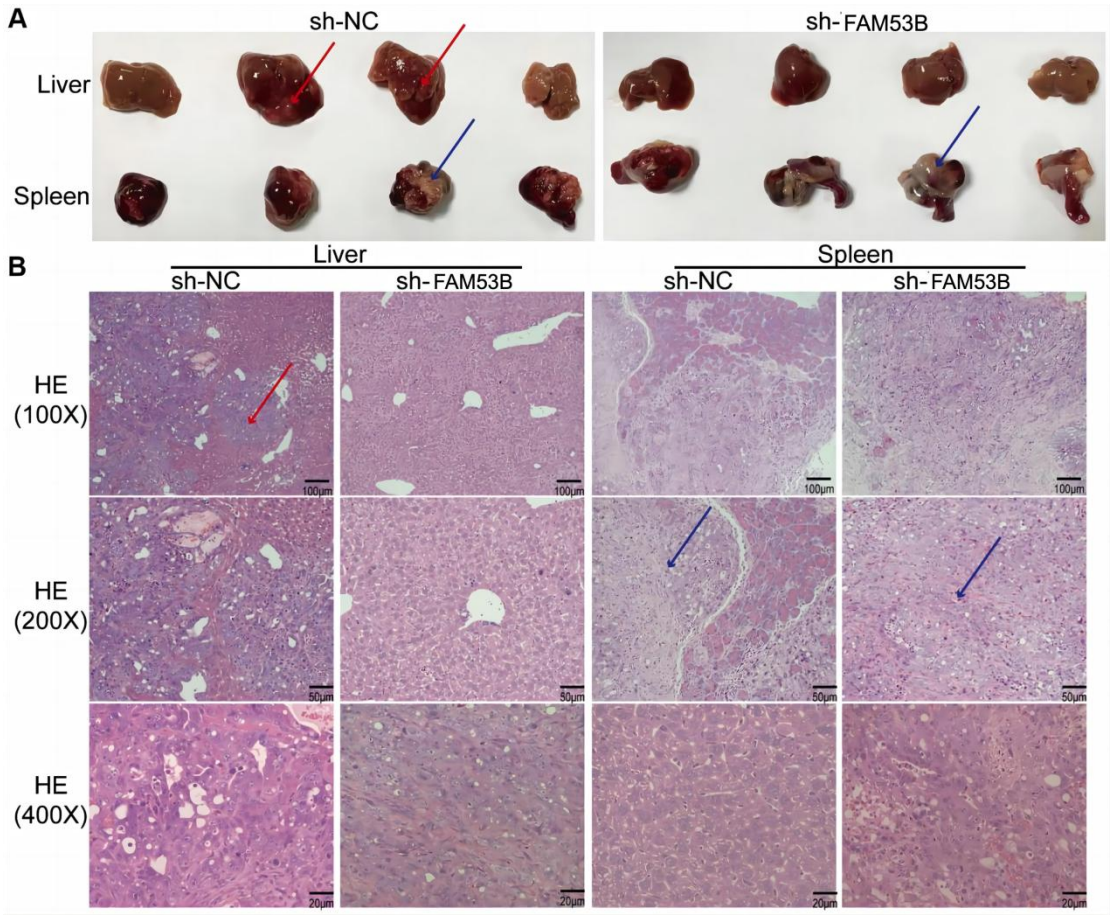

Figure 8. FAM53B induces the metastasis of pancreatic ductal adenocarcinoma

(A) Metastasis model was established in the spleen of nude mice. (B) HE staining.

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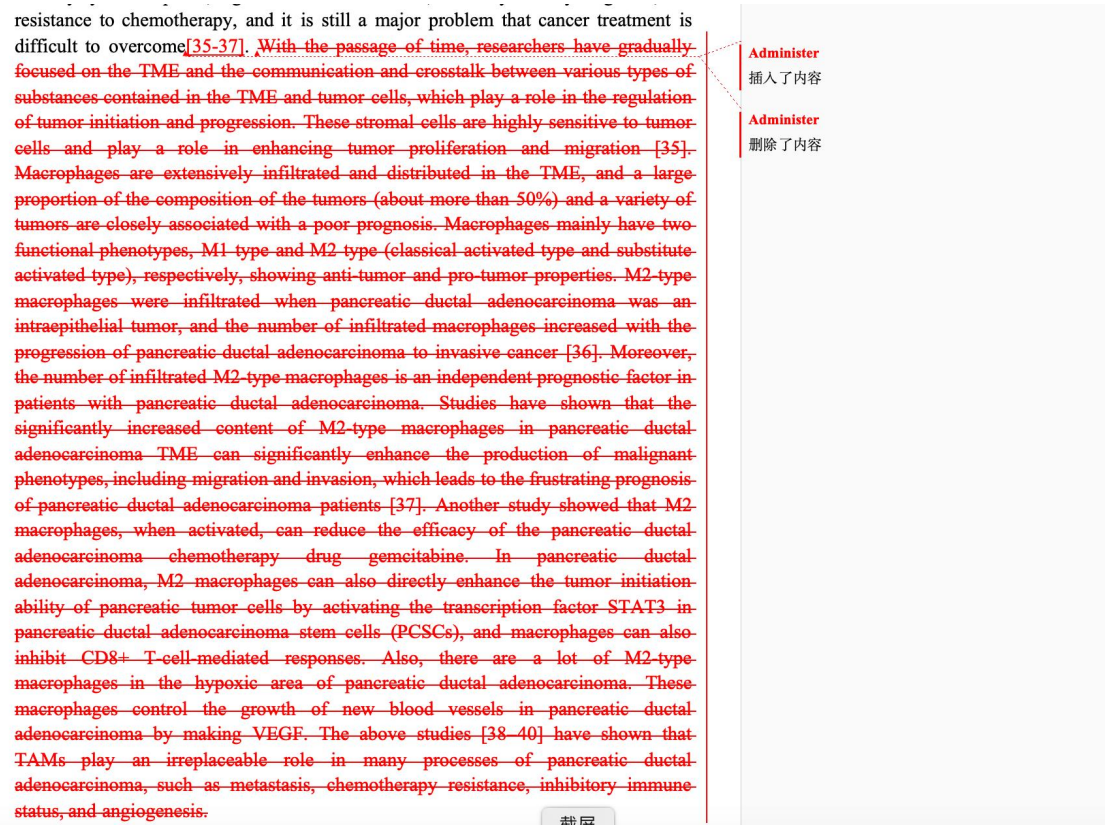
The length of the revised Discussion section was still long. The authors have to narrow down the point of discussion.

Reply 2. Thank you for your advice.

I have made appropriate cuts to the discussion section of this manuscript according to your comments. Thank you again for your suggestions.


resistance to chemotherapy, and it is still a major problem that cancer treatment is difficult to overcome[35-37]. With the passage of time, researchers have gradually focused on the TME and the communication and crosstalk between various types of substances contained in the TME and tumor cells, which play a role in the regulation of tumor initiation and progression. These stromal cells are highly sensitive to tumor cells and play a role in enhancing tumor proliferation and migration [35]. Macrophages are extensively infiltrated and distributed in the TME, and a large proportion of the composition of the tumors (about more than 50%) and a variety of tumors are closely associated with a poor prognosis. Macrophages mainly have two functional phenotypes, M1 type and M2 type (classical activated type and substitute activated type), respectively, showing anti-tumor and pro-tumor properties. M2-type macrophages were infiltrated when pancreatic ductal adenocarcinoma was an intraepithelial tumor, and the number of infiltrated macrophages increased with the progression of pancreatic ductal adenocarcinoma to invasive cancer [36]. Moreover, the number of infiltrated M2-type macrophages is an independent prognostic factor in patients with pancreatic ductal adenocarcinoma. Studies have shown that the significantly increased content of M2-type macrophages in pancreatic ductal adenocarcinoma TME can significantly enhance the production of malignant phenotypes, including migration and invasion, which leads to the frustrating prognosis of pancreatic ductal adenocarcinoma patients [37]. Another study showed that M2 macrophages, when activated, can reduce the efficacy of the pancreatic ductal adenocarcinoma chemotherapy drug gemcitabine. In pancreatic ductal adenocarcinoma, M2 macrophages can also directly enhance the tumor initiation ability of pancreatic tumor cells by activating the transcription factor STAT3 in pancreatic ductal adenocarcinoma stem cells (PCSCs), and macrophages can also inhibit CD8+ T-cell-mediated responses. Also, there are a lot of M2-type macrophages in the hypoxic area of pancreatic ductal adenocarcinoma. These macrophages control the growth of new blood vessels in pancreatic ductal adenocarcinoma by making VEGF. The above studies [38-40] have shown that TAMs play an irreplaceable role in many processes of pancreatic ductal adenocarcinoma, such as metastasis, chemotherapy resistance, inhibitory immune status, and angiogenesis.

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studies to elucidate the exact mode of action of FAM53B during the transfer process. We speculate that FAM53B may play a role by influencing macrophage activity, cell migration, and the tumor microenvironment, but the specific mechanism needs to be further explored[43].

~~On the other hand, exosomes from macrophages can also change the properties of tumor cells, which can change some of the way cancer cells behave biologically. With the help of exosomes they make, M2-type macrophages help stomach and colon cancer cells spread by making it easier for tumor cells to invade and move around. M2-type macrophage-derived exosomes may also promote angiogenesis and growth by targeting E2F2, further enhancing the malignant behavior of pancreatic ductal adenocarcinoma [43].~~ Our study shows that although FAM53B-mediated polarization of M2 macrophages does not have a direct effect on the proliferation and apoptosis of pancreatic ductal adenocarcinoma cells, this does not exclude the possibility of its involvement in the metastasis process. ~~In exploring why FAM53B affects pancreatic cancer metastasis but not proliferation, we can explore several possible explanations: First, FAM53B may affect the ability of pancreatic ductal adenocarcinoma cells to metastasize through other pathways, such as affecting cell migration, invasion, and changes in the tumor microenvironment, which may play an important role in the metastasis process without directly affecting cell proliferation and apoptosis. Second, this difference may be due to complex interactions between pancreatic ductal adenocarcinoma cells and macrophages.~~ FAM53B may mainly affect the activity and polarization of macrophages, thereby regulating their function in the tumor microenvironment, but not directly affect the proliferation and apoptosis of pancreatic cancer cells. In addition, this difference may be related to the specificity of cell signaling pathways. As a regulatory factor, FAM53B may be more likely to take part in certain signaling pathways or control certain cell functions without having an effect on cell proliferation and apoptosis. However, it is very important in controlling the metastasis process.

At the same time, exosomes from M2 macrophages can carry certain mirnas.

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The content of the first paragraph of the Discussion section appeared to be “Introduction” which was not based on the present results. For my feeling, one of the interesting points of discussion might be the different effect of FAM53B on pancreatic cancer from colorectal cancer and liver cancer.

Reply 3. Thank you for your advice.

In the first paragraph of the manuscript, there are references to the introduction part and the introduction part of the preface, and we have also made relevant content deletions to avoid repetition of the content. However, it is still necessary to mention the introduction part of the manuscript a little, so as to better connect the discussion part below and be more conducive to readers' reading and understanding. Thank you again for your valuable advice.

Minor concerns: English proofreading is still required.

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Reply 4. Thank you for your advice.

We also appreciate your suggestions. We have sent the revised manuscript to Professor Chen Xiaoyuan of the National University of Singapore for professional and English language revision so that the full text can be read more smoothly. Thank you again for your valuable suggestions.