Author’s response to reviews

Title: Helicobacter-induced Gastric Inflammation Alters the Properties of Gastric Tissue Stem/Progenitor Cells

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Author’s response to reviews:

Response to Reviewer’s comments:

Thank you for reviewing our manuscript and providing us with positive comments. The insightful suggestions have contributed in helping to greatly improve our paper. We have addressed each of the comments through the following changes to the manuscript (in red; where the changes have been made to the manuscript).
Major

1. Please describe in the text why gastric corpus organoids were generated from H. felis infected mice whereas antral organoids were generated from MNU treated mice when the MNU model was used as a positive control (page 7 line 3). These regions are very different in terms of histology, function and response to H. felis infection and putative stem cell markers are also known to have different abundances in these different regions. For instance, villin is thought to label a small subset of stem cells and these are predominantly observed in the gastric antrum.

- Thank you for your excellent comments. We completely agree with your points that the gastric corpus and antrum are quite different regions in terms of stem cell markers, biological function, and response to H. felis infection. The reason why we used either corpus or antrum differently was that each part was the susceptible region of each mice model. As for H. felis infection model, both severe gastric inflammation and SPEM (spasmolytic peptide expressing metaplasia) were both found mainly in gastric corpus. On the other hand, in MNU model gastric tumors were only found in gastric antrum, but not in corpus. Since this study focused on gastric stem cell property, we chose the gastric corpus in H. felis model, and the antrum in MNU model, respectively.

2. Please include the sex of C57BL/6 mice used for H. felis infection/organoid studies. There are known and well documented sex and background strain differences in susceptibility to pathology with this model.

- We used male mice for all the experiments of H. felis infection study and MNU chemical carcinogenesis model. There were some reports demonstrating that male mice were more susceptible to carcinogenesis in mice (Gastroenterology. 2003 Jun;124(7):1879-90.).

3. In the mouse model section, it is not clear as to whether the MNU study was first conducted in C57BL/6 mice to generate organoids prior to subcutaneous injection into NOD/SCID mice or whether tumors were generated in NOD/SCID mice. Please clarify in text. Page 6 line 16 suggests male mice were used and page 10 line 14 suggests female mice were used. Were organoids generated in males and grafted into females? Was this the same for H. felis studies?

- Thank you for your comments. We first conducted MNU study in C57BL6/J to generate organoids prior to subcutaneous injection into NOD/SCID mice. We added the text on this in
Methods. In terms of mice gender, we used male mice to generate organoids, and grafted into female NOD/SCID mice for both MNU and H. felis studies.

4. The methods of organoid analyses have not been fully described. Please include descriptions of how organoid size was measured; e.g. from how many fields of view, original magnification, how many replicates, software used.

- Thank you for your comments. When we measured the organoids’ size, we viewed 10 fields of each well by the original magnification of 100X. 5 replicates was performed per group, and the software we used was EVOS®XL Core Cell Imaging System (ThermoFisher Scientific) and Microsoft Excel to calculate the average number and size of organoids. We added the text in Methods.

5. It is not clear from this manuscript whether organoids were passaged or used immediately following their establishment. A clear indication of the experimental design is needed. There is suggestion from the literature that parietal cell loss occurs following passage of gastric organoids unless they are co-cultured with a myofibroblast feeder layer (Schumacher et al J Physiol. 2015 Apr 15; 593(Pt 8): 1809-1827). Parietal cell atrophy is also well characterized following H. felis infection in vivo.

- Thank you for your comments. We used organoids 4 days of culture, because the longer we kept culturing the organoids, the more differentiated they became. We didn’t use any feeders in our experiments to exclude the contamination of those cells in the analysis.

6. Were antibiotics used in organoid growth medium? Have the authors determined whether extracted gastric glands from H. felis infected mice remain infected during gastric organoid culture or whether the isolation and growth conditions ex vivo eliminate these bacteria? In the latter case, how are these organoids different to those generated from H. felis infected but later eradicated mice? Some indication of infection status of these organoids is needed.

- Thank you for your comments. To exclude the effect of bacterial/fungal contamination to the organoid phenotype, we used Penicillin-streptomycin (100X; Invitrogen, cat. no. 15140-122) in organoid growth media. Since culture media have few nutrition for bacteria to survive, we didn’t see any live bacteria in the dish and we considered that the organoid growth condition with these antibiotics was enough to eliminate these bacteria. We added these contexts in the text.
7. Figure 2B shows that the number of organoids generated from H. felis infected mice is greater than that from untreated mice. Please indicate how this experiment was standardized. There is great variation in number of glands extracted from stomachs and how they are fragmented regardless of treatment. The numbers presented per stomach seem remarkably low.

- Thank you for your comments. Since it was too difficult to count the number of glands per mouse, we used half of the stomach for each mouse to establish the organoids. In this experiments, we used five same aged- male mice to generate the organoids. We added the comment in the text.

8. The authors describe an increased organoid size and increased Ki67 staining (page 13, lines 8-10) however, this data is presented as 'data not shown' with no indication of quantification or justification for this statement. Please include the data and indicate in the methods how this data was generated.

- Thank you for your comments. Based on your comments, we re-analyzed and re-counted the size and Ki67 positive cells in organoid established from H. felis infected and control mice. As a result, we could not see the any increasing in terms of the size and number of positive cells for Ki67. We omitted this statement regarding size and Ki67 staining of organoids in this part.

9. The authors suggest that single organoids are derived from a single stem/progenitor cell (page 14 line 2) however, this depends on culture method used. The authors have not generated single cell gastric epithelial extracts but have generated organoids from individual glands, each of which contain several stem cells all of which could continue cycling. Please amend this paragraph.

- Thank you for your great suggestion. As you pointed out, since we generated the organoids after dissociating gastric glands into a single individual gland, not a single cell, each organoid could have contained several tissue stem/progenitor cells. We omitted this statement in the text.
10. The authors measured cytokines in organoids generated from infected mice and those post eradication however, on page 16 line 2 they suggest that this was conducted in organoids infected and post eradication which is not strictly true. Please adjust to read accurately.

- Thank you for your comments. As you pointed out, we first established the organoids from each mouse (infected or eradicated), and then measured cytokines in organoids. We amended these statements properly.

11. Please add DCLK1 data presented in supplemental figure 1 to the main body of the manuscript including how many organoids were assessed and how this was analyzed.

- Thank you for your suggestion. After establishing the organoids, we fixed them and stained with antibody for DCLK1, and viewed the positive cells in organoids. We viewed 20 organoids from 3 mice in each group. In fact, most of organoids contained only one DCLK1 positive cells, but some organoids contained more than one (2 to 4) DCLK1 positive cells in organoid established from H. felis infected mice compared with non-infected mice. With this reason, we made this statement in the results (Figure 4D).

12. Fig 4D shows gastric organoid size 3 days post cytokine stimulation vs control. I find it hard to believe that these organoids are only 6-8μm presumably (clarification in methods needed) in diameter. Single epithelial cells would be roughly this size and organoids contain several 100 to several 1000 cells. Please check size (diameter?) calculations.

- Thank you for your correction. We reviewed the photos and data of organoids, and amended the Y-axis properly.

13. Have the authors compared the phenotypic changes identified in organoids generated from H. felis infected and H. felis eradicated mice with organoid infected with H. felis in vitro? Are similar phenotypic changes observed in both models?

- Thank you for your great suggestion. Unfortunately, we have not been successful with that experiments. In microscopically, there were no phenotypic changes in organoids established from each infected- or uninfected mouse. When we tried to co-culture H. felis with organoids, most organoid didn’t look healthy, and didn’t grow well enough to analyze. In addition, our purpose of this study was to elucidate the changes of tissue stem/progenitor cells in vivo before and after H. felis infection as well as after eradication. Another reason we have not been successful that in vitro infection experiment was that we didn’t have enough
 technique to inject H. felis into the inside lumen of the organoid where apical side of epithelial cell located. In addition, the eradication of H. felis in vitro was difficult and has not been established.

Minor

1. Abstract: please use consistency when describing Helicobacter felis infection and use Helicobacter felis in full at first use.
   - Thank you for your comments, and we amended it.

2. Please add the concentration and volume of H. felis administered to mice (page 7 line 11)
   - We added the concentration and volume of H. felis.

3. Please describe the route of administration of antibiotics (page 7, line 17)
   - We administered antibiotics for eradication orally.

4. Please add concentration of N-acetylcysteine (page 8, line 15)
   - We added the comments that we added final 0.5mM of N-Acetylcysteine.

5. Fig 1B: I am not convinced by the images that there is an upregulation of Tff2 and Muc2 over the course of infection. Parietal cells look positively stained particularly in the Tff2 control tissues. Additionally, please check the magnification of 3M DCLK1 image and include scale bars on figures.
   - We reviewed our staining and exchanged the images properly.

6. I am unclear as to how E-cadherin IHC allows the confirmation of a single layer of epithelial cells (page 13, line 4). Please include data not shown data.
- Thank you for your comments. We have checked the single layer of epithelial cells by two different ways; at first, we confirmed the single layer by directly looking at the organoids under confocal microscopy. Second, we used the immunocytochemistry against a protein that strongly expressed in epithelial cells (E-cadherin in this case) easy to detect the layer of the organoids.

7. Fig 2D: The authors suggest that the Muc4 expressing region is expanded in gastric corpus glands between 3 months, 6 months and 12 months of H. felis infection, it is difficult to appreciate this from the 3 month image as this largely contains lymphoid cells. Please show a region containing mainly epithelium as for 6 months and 12 months. How was this quantified?

- Thank you for your comments. We reviewed our figure and amended properly. Since mucin protein express linearly along with the gland, quantification was too difficult to perform, and only tendency was demonstrated in this data.

8. How were organoids prepared and quantified for subcutaneous grafts?

- Thank you for your comments. First, we cultured organoids on Matrigel for 4 days as same as in vitro experiments. Then, we digested Matrigel with Cell Recovery Solution (Corning™ 354253), collect and centrifuge organoids, and re-suspended in PBS until injection. We added these sentences in the text.

9. Why is one paragraph in the discussion underlined? - the last sentence of this paragraph is particularly not well phrased.

- Thank you for your comments. The underline was mistake and was from former submission (BMGE-D-15-00096). I’m sorry for the confusion, and we deleted underline, and also rephrased the last sentence of that paragraph.

10. Isx is discussed in the discussion section however, the only previous mention of this gene was from the initial cRNA microarray. Have the authors conducted follow up studies by qRT-PCR as they have for other genes?
- Thank you for your comments. We have conducted follow up studies by not only qRT-PCR, but also other experiments. Since those experiments were published ahead (J Gastroenterol. 2016 Oct;51(10):949-60), we could not include those data in current manuscript.

11. I am not clear as to how the top paragraph on page 21 is relevant to the data presented in the manuscript. I.e. how are Sall4, Klf5, Cdx1, NFκB related to any of the genes identified in presented data?

- Thank you for your comments. We agree with you and omitted this paragraph.

Soumita Das (Reviewer 2): The paper by Shibata et al studied the possible mechanism of gastric carcinogenesis mediated by chronic inflammation after Helicobacter felis infection. They showed that the infection increased the number of tissue stem/progenitor cells, which acquired stemness properties, and altered the direction of differentiation toward intestinal metaplasia to cancer. Interestingly, they used three mice models. Also, the addition of organoids in this work has added an extra advantage. The authors responded point by point to the reviewers' comments. This manuscript is important to know the mechanism of gastric cancer. The authors should consider the following topics:

1) H. felis infection is low in human compared to H. pylori infection. They should discuss in the paper about the physiological relevance of this work in human diseases and after H. pylori infection

- Thank you for your suggestion. We added the discussion about the physiological relevance of this work in human diseases and after H. pylori infection.

1) The culture condition of the strain of Helicobacter felis should be included

- Thank you for your comments. We included the culture condition of the strain of Helicobacter felis.

Kequn Xu (Reviewer 3): In the present study, the authors demonstrated that, as one of the possible mechanisms of gastric carcinogenesis, chronic inflammation induced by Helicobacter
pylori infection can increase the number of tissue stem/progenitor cells, and alter the properties of stem cells toward intestinal metaplasia to cancer. An organoid culture system combined with a Helicobacter pylori-infected gastric cancer model and xenograft model would enable to identify cancer-initiating cells and investigation of inflammation-associated gastric carcinogenesis. You work is of interest but there are some points that need to be addressed.

1. At present, "Helicobacter" should be "Helicobacter pylori ".
   - We amended.

2. In Page 1 Line 16, if does "SPEM" mean abbreviation "spasmolytic polypeptide-expressing metaplasia"? It's not clear.
   - We made it clear by using quotation.

3. What's the difference between tissue or cancer stem cells, progenitor cells or cancer initiating cells? When you mention stem/progenitor cells, you'd better add "tissue" or "cancer" before them because they have different meaning.
   - Thank you for your great point. We meant to be “tissue” stem/progenitor cells. We added it in appropriate part.

4. When you mention "The expression levels of TFF2, MUC2, CD44, DCLK1, and VILLIN in H. felis-infected 12 mice were significantly higher than those in uninfected control mice", it is suggested to make it quantitative rather than just descriptive.
   - Thank you for your suggestion, and we counted the positive cells for each protein, and inserted the data as Figure 1C.

5. After H. felis eradication, the number of cells positive for Villin was significantly reduced while the mRNA expression of Villin in organoid was not significantly down-regulated. Could you give some possible explanation for such phenomenon?
Thank you for your great comments. We indeed showed that the mRNA expression of Villin in organoid was not significantly down-regulated. Possible explanation about this was; i). There could be different mechanism of expression between mRNA and protein, i.e. even though mRNA was expressed, posttranscriptional modification could reduce the expression of Villin, or ii) simply due to the number of mice analyzed was too small to see the statistically significant difference.

6. In Page 16 Line 14, you mention "…, suggesting that cytokine stimulation may play a role in inducing a genetic throwback from matured cell to the stem/progenitor cell phenotype". Is there any possibility that inflammation cytokines induce the transdifferentiation of stem/progenitor cells rather than a genetic throwback from matured cell to the stem/progenitor cell phenotype, which in turn accelerating proliferation?

- Thank you for your great suggestion. There were several reports that cytokine stimulation could induce the transdifferentiation of tissue stem/progenitor cells (Cabillic et al. Gastroenterology 2016). As for retrotransdifferentiation, for example, in hepatocellular carcinogenesis, retrotransdifferentiated of hepatocytes was observed through the activation of NF-kB, a key inflammatory regulator (Dubois-Pot-Schneider H, et al. Hepatology 2014). In mammary carcinogenesis, TNF-alpha exposure and SNAI2 overexpression induced partial nuclear localization of β-catenin, promoting the acquisition of a stem cell-like phenotype (Mani SA, et al. Cell 2008;133:704–715). With this regard, transdifferentiation from stem progenitor cells to non stem-cell like cells (matured cells), several reports showed that blocking of inflammatory signaling such as NF-kB, could selectively deplete cancer-stem cells, or prevent the cytokine induced EMT and stem-like features (Marquardt JU, et al. J Hepatol 2015, Han D, et al. Oncotarget 2015).

7. You mentioned in the discussion that "The mechanism how matured epithelial cells started to express stemness associated genes remains unclear." To my knowledge, in 2004, Houghton demonstrated that bone marrow-derived cells (BMDCs) might migrate from blood circulation to stomach and possess plasticity to transform into gastric epithelial cells as a result of inflammation, which may further contribute to gastric neoplasia after epithelial metaplasia and dysplasia. What's your opinion of such synopsis?

- Thank you for your great question. We have known about the Houghton’s paper, and there were some other papers supporting their data that bone marrow cells were recruited to the stomach (Varon et al. Gastroenterology 2012;142:2,281–291). We cannot completely exclude the possibility that BMDCs are the origin of stomach cancer. On the other hands, since iPS concept was now established by Shinya Yamanaka, even matured differentiate
epithelial cells could become immature/undifferentiated cells, such as highly proliferating cells or cancer cells. In the setting of stomach, we and others reported that Mist1 positive gastric stem cells are one of the candidate cells of origin for normal and neoplastic epithelial cells (Hayakawa et al. Cancer cells 2015;28:800-814). Given that, we need further experiments to confirm that which cells mainly contribute to gastric neoplasia in human stomach.

Guifang Xu (Reviewer 4):

This article by Shibata et al want to clarify the changes in epithelial stem/progenitor cells after chronic inflammation. This is an interesting research. The three-dimensional gastric epithelial cell culture system has been utilized. However, the precise mechanism has not been clarified. In this study only explained some phenomenon by mRNA expression and IHC analysis. According to current results, I think the authors cannot come to the conclusions.

Major

1. The 3D organoid culture was from mice either infected, treated with MNU, or untreated control after 4 days of culture, then, organoids were injected subcutaneously into mice and the tumorigenicity and histological changes were analyzed after 2 months (page 7 line 3-7). Only the 4 days culture is enough to form organoid? We know the 3D organoid culture from the tumor tissue need long time can do the further research.

- Thank you for your comments. As for 3D organoid culture, we surely used the organoids after 4 days of culture. At 4 days, we observed that organoids were formed and big enough to recognize. But thereafter, the size of organoid was not dramatically changed, and some organoid started to differentiate and collapsed into 2D. In order to avoid the differentiation or collapse, we used organoid after 4days culture.

2. In the methods about" 3-dimentional organoid culture of gastric epithelial cells", the mice were killed, the mouse gastric corpus was removed and cut into approximately 1-mm2 pieces. The gastric epithelial tissues were washed only by PBS, during the procedure, any antibiotics has not been used, how to avoid the cell contamination? In the previous study of prostate cancer, primocin was used during the cell culture [Unno K et al. Oncotarget. 2017 Apr 19;8(31):51264-51276.].
- Thank you for your comments. There was a same comment from the other reviewer. To exclude the effect of bacterial/fungal contamination to the organoid phenotype, we used Penicillin-streptomycin (100x; Invitrogen, cat. no. 15140-122) in organoid growth media. Since culture media have few nutrition for bacteria to survive, we didn’t see any live bacteria in the dish and we considered that the organoid growth condition with these antibiotics was enough to eliminate these bacteria. We added these contexts in the text.

3. This study only examined the stem cell markers such as Cd44, Dclk1..., only the mRNA expression and IHC analysis have been analyzed. Just like the author explained the gastric cancer stem cells marker is not very specific. So I think the following conclusion

"chronic inflammation induced by Helicobacter infection increased the number of tissue stem/progenitor cells, which acquired stemness properties, and altered the direction of differentiation toward intestinal metaplasia to cancer." cannot been drown (page 3 line 7-9).

- Thank you for your great suggestion. We agree with your comments, and our findings were not enough to draw that conclusion. We changed the sentences as follows; “chronic inflammation induced by H. felis infection increased the number of tissue stem/progenitor cells and the expression of stem cell markers. These findings suggest that chronic inflammation may alter the direction of differentiation toward undifferentiated state and that drawbacks may enable cells to redifferentiate to intestinal metaplasia or neoplasia."

4. Figure 2D should select a picture which can show the whole area, not the regional part. I think the MUC4 expression trend may be clear and more convincing.

- Thank you for your comments. We reviewed our specimens, and amended the picture showing the whole area.