Response Letter

We would like to thank the Editor and Reviewers for their evaluation of our manuscript entitled "*Expression of the methylcytosine dioxygenase TET-2 and Cx43 in inflammatory bowel disease and colorectal cancer*".

Below is a point-by-point response to the queries and concerns raised by the Reviewers.

Changes are tracked in the manuscript.

Reviewer ID 05775860 MINOR REVISION

The manuscript entitled "Expression of the methylcytosine dioxygenase TET-2 and Cx43 in inflammatory bowel disease and colorectal cancer" reports the expression levels of Cx43 and TET-2 in IBD and CRC by examining cell lines, DSS-induced colitis mouse model and patient samples. The authors used media collected from PMA- and LPS-activated THP-1 cells for colon cell culturing, to create an inflammatory milieu. Moreover, the authors generated HT-29 Cx43D (highly expressing Cx43) and HT-29 Cx43- (down-regulation of Cx43 mRNA) cells to investigate cell phenotype and gene expressions. Various independent methods were applied to support the conclusions. In addition, the data are well presented.

The below lists my suggestions that the authors may consider.

1. All the H&E and immunofluorescence images should have scale bars.

Answer: Thank you for drawing our attention to this detail. All micrographs now have scale bars, but some were indicated on the micrographs itself and others in the figure legend. This has now been standardized throughout.

2. What factors may cause the expression differences of Cx43 and TET-2 between human samples and cell/mouse models?

Answer: Cell and mouse models are usually more rigorously controlled than samples collected from humans with different genetic and clinical backgrounds. In this study, TET-2 expression was upregulated when cultured cells or animals were subjected to inflammation, a parameter that was also tightly controlled experimentally. In contrast, in human patients' tissue sections with ulcerative colitis, the extent of inflammation and the timing of the biopsy varies from one patient to another, which may affect TET-2 expression at different times in inflamed tissues and in tissues where malignancy has manifested. This study was initiated *in vitro*, followed by an established animal model of colitis. The exploratory use of human archived tissue samples was to assess and validate our findings. The complexity and the sparsity of tissues with identical features precluded us from a more rigorous evaluation of TET-2 levels in ulcerative colitis/adenocarcinoma. We now have plans to collect fresh tissues (from surgical resections), where patient characteristics are better controlled, from patients in different categories of inflammatory bowel disease (IBD) and whose colorectal cancer may have been linked to long-standing IBD.

3. The authors may consider to present a schematic figure for readers to understand the roles of Cx43 and TET-2 expressions in IBD and CRC. How may TET-2 regulate expression of Cx43?

Answer: Cx43 levels are known to be upregulated under inflammatory conditions; however, Cx43 is also considered a tumor suppressor gene and is downregulated in many cancers. TET-2 is a demethylating enzyme, which translocates to the nucleus after being synthesized and matured in the cytosol. In this study, we explore the expression levels of TET-2, in the context of colon inflammation. Our working hypothesis is that a trigger must cause the down regulation of Cx43 expression and deprive the cells from its tumor suppressor function. This prompted us to explore and attempt to unveil molecular players upstream of Cx43 expression, such as epigenetic regulators. TET-2, a demethylating enzyme, has been particularly evaluated under inflammatory conditions and is thought to play dual roles in attenuating or exacerbating inflammation in different cell and animal models. This study relied on the published literature on Cx43 and TET-2 in colon inflammation and colorectal cancer and is at the core of our hypothesis. However, further research (underway) will better establish the potential molecular link between TET-2 and Cx43 expression.

4. The additional File 1 cannot be found in the submitted files.

Answer: This was an overlooked mistake. It is now added to the submitted files.

5. The information of antibodies, including companies and catalogs, should be presented in the materials and methods section.

Answer: This information is now included in the manuscript.

Reviewer ID 05262508 MINOR REVISION

This study shows the expression of CX43 and TET2 in intestinal inflammation and discusses the expression of CX43 and TET2 in intestinal inflammation. The authors attempted to verify the relationship between CX43 and TET2 in intestinal inflammation in vivo and in vitro, and provide new targets for clinical treatment. In this paper, part of the mechanism is studied through experiments, but the mechanism research is not deep enough. Actually, the mode of action and pathway between CX43 and TET2 is not involved. This article can further explore the relationship between TET2 and intestinal inflammation.

Answer: This study reports on the expression levels of TET-2 in colon cells modified to express different levels of Cx43. TET-2 is a demethylating enzyme, which translocates to the nucleus after being synthesized and matured in the cytosol. We have explored the expression levels of TET-2, in the context of colon inflammation, which heavily modulates Cx43 expression. In fact, cells with active inflammatory response seem to upregulate Cx43. However, Cx43 is regarded as a tumor suppressor and is downregulated in many cancers. We undertook this study to decipher the molecular players upstream of Cx43 expression, such as epigenetic regulators; like the demethylating enzyme TET-2. This enzyme has been particularly evaluated under inflammatory conditions and is thought to play dual roles in attenuating or exacerbating inflammation in different cell and animal models. We hypothesize that TET-2, a general

demethylating enzyme, epigenetically regulates the expression of several genes involved in colon inflammation and in colon tumorigenesis among which is Cx43. More in depth mechanistic analyses are underway, as we modulate TET-2 expression in cancer cell lines and evaluate Cx43 expression.

Specific comments

This study shows the expression of CX43 and TET2 in intestinal inflammation and discusses the expression of CX43 and TET2 in intestinal inflammation. The authors attempted to verify the relationship between CX43 and TET2 in intestinal inflammation in vivo and in vitro, and provide new targets for clinical treatment. In this paper, part of the mechanism is studied through experiments, but the mechanism research is not deep enough. Actually, the mode of action and pathway between CX43 and TET2 is not involved. This article can further explore the relationship between TET2 and intestinal inflammation.

Answer: Thank you for a thorough evaluation of this study. In the responses to the specific queries below, we hope to ease your concerns, as we share with you some preliminary data exploring the mechanism of action behind TET-2 and Cx43 modulation in inflammation.

Suggestions

1. line 632, we hypothesize that the demethylating TET-2 enzyme might promote the expression of tumor-suppressor genes (among others, Cx43) in the inflamed colon.

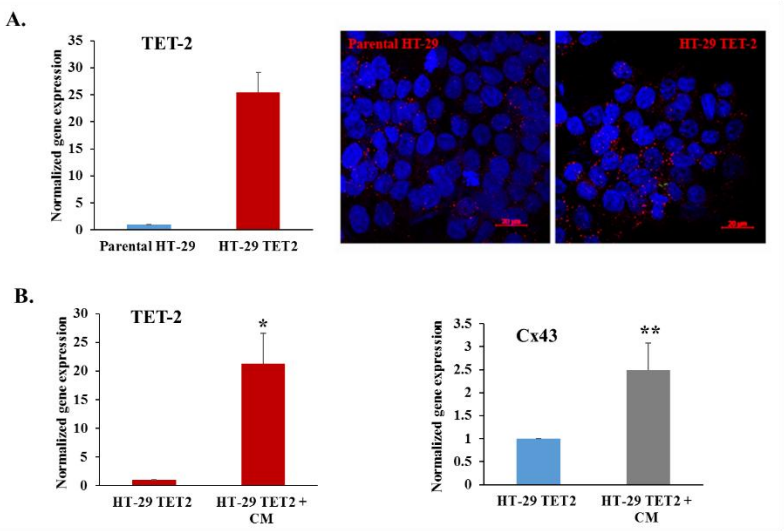
Is there convincing evidence for this hypothesis? No, this paper did not contain the mechanism study, only detect the expression of TET2 and CX43. Therefore, I strongly suggest that the authors do some experiments to explore the mechanism. Does TET2 directly or indirectly regulate the expression of Cx43? Through demethylation? Or another way?

Answer: TET-2 is a demethylating enzyme with no other reported function beyond the epigenetic regulation of gene expression. As reviewed in our manuscript, TET-2 has been firmly implicated in inflammatory processes, potentially through the regulation of expression of pro- and anti-inflammatory genes. This study is an initial work, the first to describe differential expression levels of Cx43 and TET-2 in IBD, through in vitro and in vivo models and in human samples from ulcerative colitis patients.

Cx43 is upregulated in several inflammatory conditions including IBD. We show modulated levels of TET-2 in cells with over- and under-expression of Cx43, and upregulation of TET-2 in all cells grown in inflammatory milieu; more pronounced in cells upregulated for Cx43. We also have no reason to believe that TET-2 is acting outside its demethylating activity, as demonstrated by changes in 5-hmC mark.

We are currently further exploring the issue where TET-2 expression levels in HT-29 cells are up-regulated. In the preliminary data below, exogenous TET-2 expression resulted in upregulated endogenous Cx43 expression upon exposure of HT-29 cells to inflammatory media.

The mechanism behind this apparent related regulation is not elucidated yet, but is underway. In addition to modulating the expression of TET-2 in HT-29 cells, global methylation studies will be performed using the in vitro intestinal inflammation model, in vivo colitis and colitis-induced colorectal cancer as well as samples from human patients.



HT-29 cells are transfected with exogenous TET-2 DNA, generating HT-29 TET2 cells. **A.** TET-2 upregulation was verified by qPCR and by immunofluorescence. **B.** HT-29 TET2 cells were grown in the presence or absence of inflammatory CM. Transcriptional levels of both TET-2 and Cx43 greatly increased in the inflammatory cell set, detected by qPCR.

2. line 709 inflammation. (B) Bar graphs show TET-2 transcriptional levels increase in all three HT-29 cellular subsets exposed to CM. Western blots of TET-2 and densitometric analysis show increased protein levels of TET-2 in CM-treated cells.

Why the expression of GAPDH is different between CM- and CM+ in group HT-29 CX43-Similarly, the reference gene expression of western blot assay in Fig1, Fig2, Fig5 are instability. Those experiments and figures need to improve.

Answer: The reservations that the reviewer expressed about the western blots pertain to the uneven nature of protein loading. Although we do assess protein concentration prior to sample preparation and loading onto electrophoresis runs, invariably we face issues of unequal loading (which is common), despite several experimental replicates and repeated runs. Hence, we probe for a "house-keeping" protein and express immune reactivity of proteins of interest relative to that of the house-keeping protein. While we do agree that equal GAPDH or β-actin bands more clearly reflect changes in the expression of proteins of interest, we are confident that quantification circumvents this technical shortcoming; especially that unequal loading was not always associated with the same sample.

3. line 695 inflammation. **(B)** Bar graphs show TET-2 transcriptional levels increase in all three HT-29 cellular subsets exposed to CM. Western blots of TET-2 and densitometric analysis show increased protein levels of TET-2 in CM-treated cells. **(C)** Immunofluorescence images showing TET-2 expression and 5-hmC mark in parental HT-29, HT-29 Cx43D, and HT- 29 $Cx43^{-}$ cells. Bar graphs in the right panel reflect mean fluorescence intensity of at least five different fields

acquired from three different experiments. Levels and activity of TET-2 increase all CM-exposed cells. Scale bar 5 µm.

The description of TET-2 expression between CM- and CM+ cells is inconformity with the figures.

Answer: We believe that the reviewer meant that the description is in conformity with the figures. We have, however, reviewed the figure legend and made sure that it matches with the data displayed.

Reviewer ID 05618903

It is known that colonic inflammation caused by excessive inflammatory bowel disease can initiate the colitis-associated cancer, but the mechanisms remain elusive. The authors set to bridge chronic inflammation and cancer onset in the colon using expression profile of the methylcytosine dioxygenase TET-2 and gap junction protein Cx43 in inflammatory bowel disease and colorectal cancer. They found that levels of TET-2 expression and activity increased under inflammatory conditions, in cells downregulating gap junctional protein Cx43 and in colon tissues from mice exposed to carbenoxolone, a pan-gap junction blocker, and then concluded that dysregulated expression of TET 2 may contribute to inflammation--associated colorectal cancer.

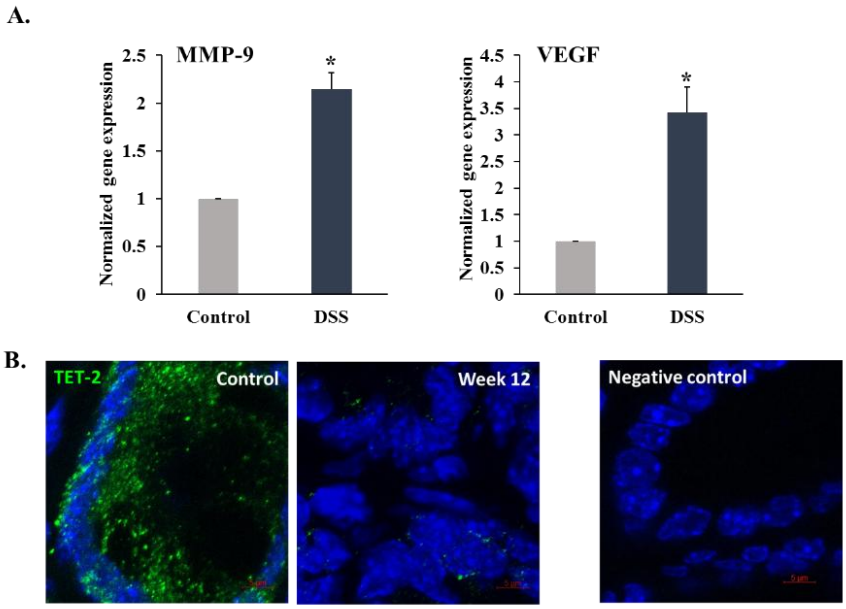
Major concerns:

1. Although the expression profile of the two targets from in vitro, in vivo and patient samples is impressive, mechanism explorations should not ever be waived.

Answer: The link between Cx43 and TET-2 is being investigated. We agree that results presented in this study do not conclude whether TET-2 directly or indirectly regulates the expression of Cx43. However, the aim was to report on the expression and activity of the demethylating enzyme TET-2 in in vitro and in vivo models of colon inflammation, under different expression statuses of Cx43, as further clarified in comment #3 below. More in depth mechanistic analyses are underway, as we modulate TET-2 expression in cancer cell lines and evaluate Cx43 expression.

2. Without colitis-associated cancer model, the conclusion was over-stated.

Answer: We have now revised the conclusion of our study and attenuated it. However, we would like to assert that the colitis-associated cancer mouse model is underway. This is part of a broader study that will also analyze samples from patients with IBD and with IBD-related colorectal cancer. Meanwhile, we show the reviewer preliminary data showing downregulation of TET-2 in the colon of a mouse from the colitis-associated colorectal cancer group.



Upregulation of EMT markers and loss of TET-2 expression in colon tissues from the colitis mouse model. Briefly, mice are injected intraperitoneally with 20 mg/Kg body weight of dimethylhydrazine (DMH; Sigma-Aldrich, Missouri, United States) at day 1 and 2.5% (w/v) of dextran sulfate sodium (DSS; Abcam, Cambridge, United Kingdom) were administered in autoclaved drinking water for 3 cycles (week 1, week 4, week 7), each cycle consisting of 4 consecutive days. DMH is a chemical agent that can initiate cancer by alkylation of DNA, thereby facilitating base mispairings, and DSS is a pro-inflammatory reagent, to generate a colitis-associated colorectal cancer model as previously described [1].

[1] Neufert, C., et al. Nat Protoc 2, 1998–2004 (2007). https://doi.org/10.1038/nprot.2007.279

3. The logic behind a special gap junction protein to be the mechanism target of intestinal inflammation is not persuasive.

Answer: We do not proclaim that Cx43 is solely responsible for the onset of intestinal inflammation. This complex process involves a plethora of players, which could together or separately modulate the inflammatory state. We and others have previously implicated inflammation with upregulation of Cx43 and gap junction-mediated intercellular communication. The analysis of Cx43 expression in this study is in the context of its up- or down-regulation in colon cell lines and the subsequent modulation of the inflammatory state. We and others have also reported that Cx43 largely exerts a tumor-suppressor role and might reverse the epithelial-to-mesenchymal transition in different cancer cell lines upregulated with Cx43. We do not imply that targeting Cx43 alone is sufficient to mitigate IBD and prevent associated tumorigenesis; we only report on the change of expression of Cx43 under inflammatory conditions, on the effect of Cx43 up- and down-regulation on inflammation, and on a potential upstream epigenetic regulator of the expression of Cx43 and several other genes involved in inflammation.