Author's response to reviews

**Title:** The role of prostaglandin E2 (PGE2) in toll-like receptor 4 (TLR4)-mediated colitis-associated neoplasia

**Authors:**

Yasmin Hernandez (yasmin.hernandez@hsc.stonybrook.edu)
John Sotolongo (jsotolongo@med.miami.edu)
Keith Breglio (keith.breglio@mssm.edu)
Daisy Conduah (dconduah@siumed.edu)
Anli Chen (Anli.Chen@mssm.edu)
Ruliang Xu (ruliang.xu@nyumc.org)
David Hsu (dhsu05@gmail.com)
Ryan Ungaro (Ryan.Ungaro@mssm.edu)
Lory A. Hayes (LHayes@med.miami.edu)
Cristhine Pastorini (stephaniechagas@gmail.com)
Maria T. Abreu (MAbreu1@med.miami.edu)
Masayuki Fukata (MFukata@med.miami.edu)

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**Author's response to reviews:** see over
April 12, 2010
Rachel Neilan, MSc
Assistant Scientific Editor
BMC-series Journals
BioMed Central
Floor 6, 236 Gray's Inn Road
London, WC1X 8HL

Dear Dr. Neilan,

We are submitting a revised version of our manuscript (1140468510350742) entitled “The role of prostaglandin E$_2$ (PGE$_2$) in toll-like receptor 4 (TLR4)-mediated colitis-associated neoplasia” by Yasmin Hernandez, John Sotolongo, Keith Breglio, Daisy Conduah, Anli Chen, Ruliang Xu, David Hsu, Ryan Ungaro, Lory A. Hayes, Cristhine Pastorini, Maria T. Abreu, and myself.

We have made changes to our manuscript by which we have addressed the comments and concerns of the reviewers. In response to the suggestions by reviewers, we have provided additional analyses of Amphiregulin protein, TUNEL assay, and Cox-2 expression in TLR4-deficient macrophages. The revised manuscript is significantly strengthened by these additional experiments and comments about interpretation of our results. We have highlighted changes to the manuscript in red text.

Given the reviewers’ initial enthusiasm of this manuscript and our specific revisions and additional data in response to their suggestions, we hope that you will find our work acceptable for inclusion in the Journal.

Thank you for your consideration,

Masayuki Fukata
Assistant Professor of Medicine
Division of Gastroenterology
University of Miami Leonard M. Miller School of Medicine
Specific Comments to Reviewer #1: Jin-Kyung Kim

The authors claim that their work demonstrated that exogenous PGE2 administration increase colitis-associated tumorigenesis via activation of EGFR signaling pathway and dysregulation of PGE2 synthesis in TLR4 KO mice. I have a few minor concerns that need to be addressed.

1. **Fig. 2B, Size-bar should be indicated.**

   As suggested, we have added appropriate size-bars to our pictures.

2. **Fig. 3C, The levels of 15d-PGJ2 of colon in experimental groups were shown by bar-graph. If the PGE2 levels were shown same pattern (not just explain in the text), the audience easily understand that PGE2 administration affects the balance between 15d-PGJ2 and PGE2. Could the author explain the reason(s) how PGE2 administration increased the level of 15d-PGJ2 in colon.**

   The reviewer makes a very important point about the balance between PGE2 and 15d-PGJ2 during active and the recovery period of the colitis. Since our experimental setting is very specific to the TLR4-deficient environment, the result we found “a differential regulation of 15d-PGJ2 in response to exogenous administration of PGE2 between the active and recovery phases of colitis” might not be directly applicable to the general roles of PGE2 and 15d-PGJ2 in colitis. As suggested by the reviewer, we have added new figures in Figure 3 to show the endogenous PGE2 production as bar-graphs. Although we do not see a difference in mucosal 15d-PGJ2 synthesis, endogenous mucosal PGE2 is significantly increased in the mice treated with PGE2 during recovery compared to the mice treated with PGE2 during acute colitis. By contrast, there is a significant increase of mucosal 15d-PGJ2 when PGE2 is administered during acute colitis, while no increase of endogenous PGE2 is observed. These results indicate that there is a stimuli that induces 15d-PGJ2 during active colitis but not during recovery from colitis and that the ratio of PGE2 vs. 15d-PGJ2 (cell-proliferative vs anti-inflammatory) prostaglandins are balanced only in the active phase of colitis. Thus our results indicate without such stimuli to induce 15d-PGJ2 production, intestinal mucosa cannot maintain the balance between PGE2 and 15d-PGJ2 during the recovery phase. We do not think that PGE2 simply induces 15d-PGJ2 based on these results but PGE2 administration during recovery from colitis enhances endogenous PGE2 as well. This has been shown in the Figure 7. We have added additional sentences in the results section (page 17 and 18), which we believe has made this point more clear to the reader.

3. **Fig. 4A, Magnification and size-bar should be indicated.**

   As suggested, we have added the magnification in the legend and a size-bar to this figure.

4. **Fig. 5A, The author showed the mRNA levels of AR to explain PGE2 regulate EGFR-signaling pathway in TLR4 KO mice. Based on their previous work (2007), they showed the protein levels of AR. How about the protein
levels in the present study?

We agree that it is important to note the protein levels of Amphiregulin in this present study. In this study, we used colon lysate samples, as we did not have the colon ex vivo culture samples that we used in our previous paper. The results of this experiment show that protein levels of AR correlate with the level of AR mRNA found in our PCR experiment. We have included this data in the text (page 19.) and Figure 5A.

Specific Comments for Reviewer #2: Naofumi Mukaida

1. There are numerous reports, which indicate the indispensable roles of COX-2-mediated prostaglandin E2 in colon carcinogenesis. The authors demonstrated that COX-2 is present downstream TLR-4. Thus, the data in the present paper seems to be confirmatory. The authors also tried to elucidate the molecular mechanisms underlying prostaglandin E2-induced acceleration of colon carcinogenesis. Low dose of prostaglandin increased the amount of amphiregulin and phosphorylated EGF receptor without enhancing cell proliferation. Thus, it is unlikely that amphiregulin and EGF receptor system can be involved in prostaglandin E2-induced acceleration of colon carcinogenesis. Thus, the authors are encouraged to provide mechanistic insights on the effects of prostaglandin E2.

We appreciate the reviewer’s comment to provide mechanistic insights on the effect of AR-EGFR interaction in PGE2-induced acceleration of colon carcinogenesis. The magnitude of the AR-EGFR interaction is what is inducing the acceleration of colon carcinogenesis. PGE2 increases the AR production, which in turn causes a phosphorylation of the tyrosine residues on the EGFR leading to the activation of a signaling pathway inducing the epithelial proliferation. It is important to note that our current study is examining the role of PGE2 in TLR4-mediated colitis-associated tumorigenesis. It is true that activation of EGFR and up-regulation of AR is not only involved in intestinal tumorigenesis but is also involved in the normal mucosal repair process. Therefore, we believe that the discrepancy in our results between AR-induced EGFR activation in cellular proliferation and in tumor development shows the differential roles of this process. While there may be more factors involved in the regulation of the different roles of AR-induced EGFR activation during the colitis and colitis-associated tumorigenesis, we believe our results demonstrate an important mechanistic insight into TLR4-mediated colitis-associated tumorigenesis. Therefore, our results are not only confirmatory but also demonstrate novel mechanistic findings regarding colitis-associated tumorigenesis. To highlight this, we added comments in the discussion section of our paper (page 23).

Specific comments

#1. Table 1. Tumor incidence in WT mice was calculated as 92.3 %, although the authors used only 7 WT mice. The calculation seems to be wrong. The range of percentage of mucosal surface with tumor is 1-40 and 1-5, in WT and PBS-treated TLR4 KO mice, respectively. However, the incidence of these two groups is not 100 %. Thus, the lower range of these two groups should
be 0.

We have reviewed these calculations and apologize for our mistake. The number of WT mice was 13. We have made the appropriate changes to the results section and Table 1.

#2. Figure 3 B and C. The authors should describe the dose of prostaglandin E2 used in these experiments. In Fig. 3C, 15-d-PGJ2 levels should be compared with untreated group.

As suggested, we added the information for the dose of PGE2 used in the experiment in the figure legend. We agree that 15-d-PGJ2 levels should be compared with the untreated group and original figure 3B has already showed basal level of 15-d-PGJ2. We have further added his information in figure 3C.

#3. Figure 6A. The used dose of prostaglandin E2 should be described.

We added the information for the dose of PGE2 used in the experiment in the figure legend.

Specific Comments to Reviewer #3: Shin Maeda
Major Compulsory Revisions

This manuscript investigated the effect of PGE2 on colitis-associated neoplasia. The result may be potentially interesting, but there is a question about experimental design and there are some key data missing from this work.

1. The authors described that no significant difference was seen in the incidence of colitis-associated neoplasia between the mice treated with PGE2 during DSS treatment and treated with PBS, and that increased mucosal PGE2 during the acute phase of colitis does not promote tumorigenesis. However, the number of the mice treated with PGE2 was 5, and only one mouse developed dysplasia. The number of mice treated with PBS was 6, and only two mice had dysplasia. These numbers seemed to be insufficient to assess. Previously the authors showed PGE2 ameliorates acute colitis in TLR4-/- mice if administered during DSS treatment in Ref.27. Why didn’t the decreased inflammation in PGE2-treated mice reduced the development CAC in this study?

We appreciate the reviewer’s comments. With the number of mice we have, we have been able to demonstrate our specific points, which include describing the role of PGE2 in the TLR4-mediated colitis-associated tumorigenesis setting. The PBS treated group was seven mice and the incidence of polyps was comparable to the TLR4-/- mice we observed in Ref. 28. While conducting more experiments would increase our sample size, we believe that our data as a whole is convincing to demonstrate the point that PGE2 administration during recovery period of colitis bypass the protection from colitis-associated tumorigenesis in TLR4-/- mice. A larger sample size would be confirmatory, but we thought this time it would not significantly contribute further to new scientific findings.
The reviewer also makes a good point regarding the role of inflammation in colitis-associated tumorigenesis. As shown in Figure 3A, the histological severity of the colitis was similar over the entire course of chronic colitis (day 56). It is of course possible that the degree of inflammation varied during DSS treatment in the AOM-DSS model; however, the end point of this experiment showed no difference in severity of inflammation among the groups examined (Figure 3A). Although some studies (Suzuki et al., Histol Histopathol. 2005, and Clapper et al., Inflamm Bowel Dis. 2008) have shown a correlation between the severity of colonic inflammation and tumor incidence in certain animal strains, it is not sure whether such a correlation is applicable to our study using C57Bl6 background mice (Suzuki et al., Carcinogenesis. 2006).

2. The authors described that the mice in the acute phase of colitis had significantly higher production of mucosal PGE2 than the mice in the chronic phase (331.0 vs. 223.8). I think that the amount of PGE2 which was administered during DSS treatment was insufficient because mucosal PGE2 increased during DSS treatment, and that relatively low dose of PGE2 may decrease the phenotypic difference. Does treatment with more doses of PGE2 during DSS influence the colitis activity or the development of CAC?

We conducted initial experiments with a higher dose of PGE2 (400 and 800 µg/day); however, the mice did not survive this treatment. Mice that received the elevated levels of PGE2 developed severe diarrhea, and died of dehydration regardless of the histological severity of colitis. Also, our dose 200 µg/day induced increased intestinal epithelial proliferation after 7 days of treatment. Based on these findings, we decided upon the dose of PGE2 given in our experiment.

3. The authors showed that PGE2 plays different roles during the acute and the chronic phases of colitis. The authors should examine whether the administration of PGE2 during or after DSS treatment affects the colitis activity by BW change, disease activity score, or histological score, and compare both administration protocols.

We appreciate the reviewer's suggestion and have added data for disease activity index score to compare both acute and recovery administration protocols (Figure 3A).

4. The authors showed the total histological score of AOM-DSS-treated mice in Figure 3A, but the authors should show details of this score, for example, the infiltration of myeloid cells and the epithelial cell damage. “NS” was described in this figure, but the difference between WT and PBS-treated TLR4-/- mice seems to be significant.

As suggested, we have evaluated the details of the histological score of the AOM-DSS-treated mice. However, there was no particular difference in inflammatory cell infiltration and epithelial damage, etc. We have added this comment in the result section (page 16). Using Prism software to calculate statistical significance, One-way ANOVA showed a P value of 0.1391, and Kruskal-Wallis test showed a P value of 0.1224 for the histological score results.
5. In Figure 3, B and C, the authors showed mucosal 15d-PGJ2 synthesis was up-regulated in day7, but not in recovery phase. However, the authors described “Exogenously administered PGE2 disturbs the balance between cell-proliferative and anti-inflammatory prostanoids during the recovery phase but not during the acute phase of colitis.” This is a little confusing. When does mucosal 15d-PGJ2 play an important role, during the acute phase or in the recovery phase?

The reviewer brings up an important point. As 15d-PGJ2 has been considered as anti-inflammatory prostaglandin, we believe that 15d-PGJ2 plays an important role in acute colitis to maintain mucosal homeostasis. However, our focus in this study is to clarify the role of PGE2 in TLR4-mediated colitis-associated tumorigenesis. Our point is that exogenous administration of PGE2 results in increase of 15d-PGJ2 when administered during active colitis, but increases PGE2 when administered during the recovery phase of colitis. This differential induction of prostanoids leads to different tumor incidence in our model. To make this point clear, we have added new figures in Figure 3 to show the endogenous PGE2 production.

6. In Figure 3, B and C, the authors should show the time course of mucosal 15d-PGJ2 synthesis in WT mice and TLR4-/- mice.

We appreciate the reviewer’s comment, as our main objective in this experiment is to study the effects of PGE2 in TLR4-mediated colitis-associated tumorigenesis. We have examined mucosal 15-d-PGJ2 synthesis in basal, day7 of DSS treatment, and 14 days of recovery from DSS treatment to have a meaningful stepwise alteration of mucosal 15-d-PGJ2. We have added 15d-PGJ2 data in WT mice as comparison.

7. In Figure 4, were these colonic sections removed from AOM-DSS-treated mice? If so, the authors also should perform BrDU assay without AOM-DSS.

We have already published BrdU data in normal WT mice and TLR4-/- mice in our previous work, Ref 27 (Figure 3B in Fukata et al, Gastroenterology 2006).

8. In Figure 5, the authors should analyze the expression of AR and p-EGFR in non-tumor epithelia and tumor section separately.

We agree that it is important to analyze the expression of AR and p-EGFR in non-tumor epithelia and tumors separately; however, we did not see the specific benefit of examining AR and EGFR in the tumor itself for the purpose of our study. There are many reports that have described, including our previous studies, that AR and EGFR activation are upregulated in intestinal tumor cells. (Ref. 35, Damstrub, Br J Cancer, 1999, Nishimura, Oncol Rep 2008, Rego, Br J Cancer 2010). Our experimental goal is to examine what happens in the process of colitis-associated tumorigenesis, and give some insight as to the mechanisms involved in tumor development during colitis-associated neoplasia. With this goal in mind, it is important to study the biological alterations in non-tumor mucosa during acute and chronic colitis, as this can reveal differing factors
involved during tumor development. While demonstrating that AR is upregulated in the tumor itself would be confirmatory to many previous studies, it does not give any mechanistic insight as to tumorigenesis in our experimental setting. Our study as looked at the surrounding, non-tumor mucosa and found that PGE2 is a key molecule found in downstream TLR4 signaling that induces the upregulation of AR expression and in turn EGFR-phosphorylation and as such, we have elucidated a potential mechanism for tumor formation.

As pointed out by the reviewer, we have added the distinction that our samples come from non-tumor mucosa and have amended our text (page 10).

9. In Figure 6A, the expression of Cox-2 was elevated in the mice treated with high-dose PGE2. Were other inflammatory cytokines, such as TNF, IL-6, or MIP2, also elevated?

We appreciate the reviewer's suggestion and have included MIP2 real time PCR data in the text (page 20).

10. In Figure 6C, the authors should include the data using TLR4-/- macrophages.

As suggested, we have demonstrated the data regarding TLR4-/- peritoneal macrophages in figure 6C.

11. In Ref.27, it was said that there was a significant decrease of apoptotic cells in PGE2-treated TLR4-/- mice compared with vehicle-treated TLR4-/- mice. The authors should include the analysis of apoptosis in this study because it has been shown that apoptosis plays an important role in the development of CAC.

As suggested, we performed TUNEL assay to demonstrate the role of apoptosis in our study. We included this data in figure 4C.