Peer Review File

Article information: https://dx.doi.org/10.21037/tcr-22-2552

Reviewer Comments

Comment 1: In Figure 2, the authors examined the contribution of PGE2-EP4 by treating the cells with PGE2 or L-902688, citing the paper (20). However, the authors previously showed that TFF3 increases PTGS2 expression in SW620 cells and PGE2 production in HCT-8 cells. In other words, the results in Figure 2 are experiments that confirm the reproducibility of previous research results.

Reply 1: We very appreciate your insightful comments. Li Yang et al. showed that inhibiting EP4 receptor reduces tumor growth and metastasis in colorectal cancer and lung cancer (Reference 20). We previously demonstrated that TFF3 promotes PTGS2 expression and PGE2 production in colorectal cancer cells (Reference 4). In this study, we reconfirmed the role of EP4 in TFF3-facilitated clonogenic survival of colorectal cancer cells by treating TFF3 knockout cells (SW620KO) with EP4 ligand PGE2 or EP4 agonist L-902688, and TFF3 overexpressing cells (TFF3OE) with EP4 antagonist ONO-AE3-208. These experiments not only confirm the reproducibility of previous research results but also highlight the potential role of EP4 in TFF3-spured clonogenic survival of colorectal cancer cells.

Changes in the text: We have modified our text as advised (see Page 10, line 212).

Comment 2: In addition, since the expression of EP4 is already low in SW620-TFF3KO cells (SW620KO) cells, experiments to treat the ligand for EP4 are too unnatural. The authors need to cite their paper (4) in the section of Figure 2 and carefully introduce their research background regarding TFF3.

Reply 2: Thank you for your insightful comments. We have introduced the research background regarding TFF3 in the section of Figure 2.

Changes in the text: We have modified our text as advised (see Page 10, line 204).

Comment 3: In addition, the author thinks it is important to show whether the exogenous expression of EP4 can compensate for the deficiency of TFF3 in SW620KO cells in Figure 3. The reviewer thinks it is not easy to compensate for the function of TFF3 just by EP4 because PGE2 is decreased. However, the reviewer thinks it is important to provide the information to the readers, and the experiments to treat the cells with the agonists for EP4 in this situation are natural.

Reply 3: Thanks for your careful evaluation. In Figure 3, we demonstrated that TFF3 regulates EP4 expression. We agree that it is not easy to compensate for the function of TFF3 just by EP4 because PGE2 is decreased. We have added some detailed information according to your suggestion.

Changes in the text: We have modified our text as advised (see Page 10, line 207).

Comment 4: Related to the previous comment, in Figure 5, the authors previously showed that TFF3 regulates STAT3 activation via CD147, and the results in Figure 5 are experiments that confirm the reproducibility of previous research results. The authors must introduce their research background regarding TFF3 and STAT3 in Figure 5. In addition, the authors should examine whether PTGER4 expression is decreased in HCT-8 CD143KO cells.

Reply 4: Thanks for your insightful comments. We have provided the research background regarding TFF3 and STAT3 in the section of Figure 5 (see Page 11, line 231). We also determined the expression of EP4 in HCT-8 CD147KO cells and found that EP4 was reduced in CD147KO cells compared with the control cells (Figure 6K).

Changes in the text: We added the data as shown in Figure 6K and described in Results (see Page 11, line 245).

Comment 5: In Figure 6, the authors examined the contribution of STAT3 in the induction of PTGER4 expression in TFF3 over-expressed HCT-8 cells. However, it is still unclear whether STAT3 regulates PTGER4 expression endogenously. The authors should examine whether mutation of STAT3 binding sites decreases reporter activity using SW620 cells.

Reply 5: Thanks for your insightful comments. We transfected SW620 cells with luciferase reporter vector containing wild type *PTGER4* promoter (-437~+1) or mutant promoter (as shown in Figure 6C), and found that mutation of predicted STAT3 binding sites led to decreased reporter activity (Figure 6E), indicating that STAT3 regulates *PTGER4* transcription endogenously.

Changes in the text: We added the data as shown in Figure 6E and described in Results (see Page 11, line 239).

Comment 6: Related to the previous comment, the authors should examine the contribution of STAT3 to PTGER4 induction in SW620 cells using Niclosamide and siSTAT3. In addition, to avoid off-target effects of siRNAs, the authors should use multiple siRNA against STAT3.

Reply 6: Thanks for your insightful comments. We treated SW620 cells with increasing amount of niclosamide and found that STAT3 inhibition resulted in reduced EP4 expression (Figure 6I). We also silenced STAT3 expression in SW620 cells with two different siRNAs targeting STAT3 and revealed that knockdown of STAT3 decreased EP4 expression (Figure 6J). These results suggest that STAT3 contributes to EP4 expression in SW620 cells.

Changes in the text: We added the data as shown in Figures 6I-J and described in Results (see Page 11, line 243).

Comment 7: The authors need to add molecular weight markers in all western blotting data.

Reply 7: We have added molecular weight markers in all western blotting data.

Changes in the text: Not applicable.

Comment 8: In Figures 1E, 2A, 2B, 4B, 4C, 6D, 6E, 6F, and 6G, because these experiments are multiple comparisons, the authors should perform one-way ANOVA as a comparison test, not a two-tailed Student's t-test.
Reply 8: Thanks for your careful evaluation. We have reanalyzed the data using one-way ANOVA.
Changes in the text: We have modified our text as advised (see Page 9, line 182).

Comment 9: In Figure 6G, there is a typo, not siNRA; it should be siRNA.
Reply 9: We are sorry for the carelessness. We have corrected the typo.
Changes in the text: Not applicable.

Comment 10: In Figure 7A, enlarged images are also needed.
Reply 10: We have provided the enlarged images in Figure 7A.
Changes in the text: Not applicable.

Comment 11: In Material Methods (Line 87-88), it is still unclear how they made SW620KO cells. Which gRNAs and Cas9 vectors did the authors use? The information is also lacking in their previous study (4). In addition, how did the authors select the clone? Is this derived from a single clone or balk?
Reply 11: Thanks for your careful evaluation. The TFF3 KO SW620 cells were generated with the CRISPR/Cas9 system. All-in-one plasmid lentiCRISPR v2 was obtained from Addgene (Watertown, MA, USA). On-target sequence was 5′-ATGTCACCCCCAAGGAGTGC-3′. HEK293T cells were used to produce lentivirus and the viral supernatant was aliquoted and stored at -80 °C. SW620 cells were seeded at 5×10^5 cells/2 mL in antibiotic free RPMI 1640 medium. Twenty-four hours later, 100 µL viral supernatant was added to the 2 mL of media on the cells and gently rocked plate to mix. At 48 hours after infection, cells were screened using puromycin for 6 days at a final concentration of 2 µg/mL. Single cell-derived clones were obtained by limiting dilution and edited single clones were verified by western blotting. We have added the information above in the revised manuscript.
Changes in the text: We have added the information in Methods (see Page 5, line 91).

Comment 12: In Material Methods (Line 126-131) and the legends of Figures 4 and 6, it is still unclear how the authors normalized the data and how they compared the results. The authors should add an explanation regarding these points.
Reply 12: Thanks for your careful evaluation. The luciferase reporter assays were performed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The relative luciferase activity was calculated by dividing results from the Firefly luciferase assay over the Renilla luciferase assay. The following equation was used to determine the normalized fold change in luciferase activity between test groups:

$$\Delta\text{Fold activity} = \frac{\text{Average (Firefly/Renilla) from each construct}}{\text{Average (Firefly/Renilla) from construct pGL3} - \text{Basic}}$$

Each construct was compared to the luciferase activity of construct pGL3-Basic (an empty vector). The normalized fold changes in luciferase activity from each experiment were averaged together, and the statistical significance determined. We have added the information above in the revised manuscript.

Changes in the text: We have added the information in Methods (see Page 7, line 151).

Comment 13: In Material Methods, the information of siRNAs is lacking. This information is also lacking in their previous study (4). Which siRNAs did the author use? By what method did they use for the treatments of siRNAs?

Reply 13: We are sorry for the lack of this critical information. Specific small interfering RNA (siRNA) and scramble control siRNAs were obtained from GenePharma (Shanghai, China). A mixture of two individual siRNAs, each aimed at a different region of the STAT3 mRNA, was used for silencing STAT3. HCT-8 and SW620 cells were seeded in 6-well plates, and the cells were transfected with 20 nM siRNAs using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The siRNA sequences were as follows: siSTAT3-1: 5′-CCACUUUGGUGUUUCAUAA-3′; siSTAT3-2: 5′-GAGCUGCAAACAACUAUAC-3′; scramble siRNA: 5'-UUCUCCGAACGUGUCACGU-3'. We have added the information above in the revised manuscript.

Changes in the text: We have added the information in Methods (see Page 6, line 115).