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Grenoble, May 5th, 2017

Dear Editor,

Thank you very much for handling our manuscript entitled " Involvement of CRF2 signaling in enterocyte differentiation" (World Journal of Gastroenterology N° 33280). We also would like to thank the reviewer for his time and comments. We were very pleased to have the opportunity to modify the manuscript along the lines suggested by the reviewer. Enclosed is a new revised version of the manuscript that takes into account the reviewer's comments. Our responses to the reviewers and a description of the changes to the manuscript are outlined below.

Reviewers' comments and Reviewer's Responses to Questions

This manuscript demonstrated that Ucn3-induced CRF2 signaling could modulate intestinal epithelial cell differentiation and epithelial cell permeability. The authors found CRF2 was associated with a poor differentiated status of IEC. Then, they proved CRF2 signaling altered the trans- and para-cellular permeability, and delayed colonic cell differentiation. In general, the work would be potentially useful to reveal the roles of CRF2 signaling in tumor progression. However, the manuscript requires some modifications before publication. More specially, the authors should adequately address my following points:

MAJOR COMMENTS
1. The authors mainly used HT-29 and Caco-2 cells to investigate CRF2 signaling in colorectal cancer cells. However, some results were only obtained from one cell line. For example, the result that CRF2 signaling increased trans-epithelial permeability was only showed in HT-29, while the result that Ucn3 decreased the mRNA and protein expression of KLF4 was only shown in Caco-2. Generally, consistent phenomena in multiple cell lines will increase reliability.

We agree with the reviewer that demonstration of consistent phenomena in multiple cell lines will increase reliability. That's why we performed most of the experiments in both HT-29 and Caco-2 cell lines. However, as explained in the manuscript, Caco-2 cells are preferentially used to study enterocyte differentiation. At 21 days post-confluence, Caco-2

cells are maximally differentiated into mature absorptive epithelial cells, both phenotypically and functionally. They form a polarized monolayer of cells that: express microvilli at their apical pole, contain digestive enzymes (such as disaccharidases or peptidases) and establish intercellular contacts with both mature AJ and TJ [44]–[46].
According to the rewiever's comment, we performed new experiments to complete figure panels in both cell lines. At first we tested the effect of CRF2 signaling on trans-epithelial permeability in Caco-2 cells. These data confirm that CRF2 signaling increases trans-cellular permeability. They are presented in a new figure 4 (Figure 4A), and accordingly described in the text.
Then, we looked at the effect of Ucn3 on mRNA and protein expression of KLF4 in HT-29 cells. Our data indicate that, as observed in Caco-2 cells, CRF2 signaling decreased both mRNA and protein expression of KLF4 in HT-29 cells too. A new figure 5B has been added and described accordingly in the text.

2. Some conclusions should be supported by proper statistical tests. For example, the result that CRF2 expression is also inversely correlated to E-cadherin expression in these cell lines should be tested in Wilcoxon signed rank test.

We agree with the reviewer that some conclusions were not supported by proper statistical tests. For this purpose and in collaboration with a statistician we have revised our statistical methods used to verify the results. The most adequate and appropriate method for our experiments consists in a One-way or two-way ANOVA according to the experiments. These modifications have been indicated in the Materials and Methods section and in the respective figure legend.
However the reviewer proposed that the result suggesting an inversed correlation between CRF2 expression and E-cadherin expression in various cell lines should be tested in Wilcoxon signed rank test. Unfortunately to our knowledge this kind of test doesn't permit to establish a correlation. Hence we performed a correlation test and a linear regression taking all independent manner values (for correlation: spearman r = - 0.8881, p value = 0.0003) (for linear regression: r2 = 0.8748; p value <0.0001). The data has been added in the text.

3. Did the reduced expression of KLF4 was indirectly induced by CRF2 activation, e.g., direct interaction, or an indirect effect? More evidences are required.

Many observations are in favor of an indirect effect of CRF2 action on KLF4 expression.
1) KLF4 expression increases during the process of cell differentiation whereas CRF2 expression decreases.
2) KLF4 expression is transcriptionally regulated during cell differentiation in both cell lines.

3) KLF4 expression is increased with the establishment of mature intercellular junctions.
4) Chronic treatment with Ucn3 compared to a single exposure ("acute stress") has more severe consequences on mRNA and protein expression of KLF4.
Our hypothesis is thus rather in favor of an indirect effect of CRF2. One possible mechanism is that by dissociating intercellular junctions Ucn3-mediated activation of CRF2 signaling could indirectly regulate KLF4 expression at both transcriptional and post-transcriptional levels. The role of intercellular junctions in the regulation of transcriptional factors involved in enterocyte differentiation has already been demonstrated for Cdx2 (Houde et al, 2001; Laprise et al, 2002). KLF4 expression is dependent of cdx2 in human colon cancer cells (Dang et al, 2001). Furthermore, we demonstrated that Ucn3-mediated cell dissociation is associated with nuclear translocation of β-ctn. It has been proposed that elevated β-ctn/Tcf signaling reduces levels of KLF4 (Flanchez et al., 2008).
This hypothesis has been further discussed in the discussion.

MINER COMMENTS
1. Did Ucn3 reduce both the mRNA and protein levels of DPPIV and AP, or only protein levels?

Ucn3-mediated activation of CRF2 signaling reduces both the mRNA and protein levels of DPPIV and AP protein levels. These data have been presented in a new figure 6 (Figure 6A), and described accordingly in the text.

2. Figure 3A compared trans-epithelial electrical resistance with or without A2b overnight before addition or not of Ucn3. However, the results of Ucn3 untreated cells were not shown. The same problems existed in Figure 4A.

The reviewer is right. A new Figure 3A showing the TEER of Ucn3 untreated cells with or without A2b has been included in the manuscript and described accordingly in the text. The same experiment has been done with caco-2 cells and included in a new figure 4 (figure 4B), and described accordingly in the text.

3. There were many mistakes in format. For example, " Cdx2, Hox, HNF, GATA4, KLF4..." should be "Cdx2, Hox, HNF, GATA4, KLF4, etc." Also, "differentiation processes that occur during organogenesis and migration along the cryptvillus axis [29]–[31]" should be "differentiation processes that occur during organogenesis and migration along the cryptvillus axis [29-31]".

We agree with the reviewer, a few mistakes were scattered in the text. We carefully re-read the manuscript and corrected them. The suggested modifications have been done accordingly. The manuscript is more accurate and complete.

We hope that you will find our revised manuscript suitable for publication in World Journal of Gastroenterology. We would like to thank the reviewers for their input as the added corrections strengthen the manuscript.

Thank you for your time and effort in considering and handling this manuscript.

Sincerely yours,

Dr Muriel Jacquier-Sarlin (PhD)