Prostaglandin E2 promotes intestinal repair through an adaptive cellular response of the epithelium

Hiroyuki Miyoshi, Kelli L. VanDussen, Nicole P. Malvin, Stacy H. Ryu, Yi Wang, Naomi M. Sonnek, Chin-Wen Lai, and Thaddeus Stappenbeck

Corresponding author: Thaddeus Stappenbeck, Washington University - St. Louis

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>28 April 2016</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>02 June 2016</td>
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<tr>
<td>Revision received</td>
<td>01 September 2016</td>
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<tr>
<td>Editorial Decision</td>
<td>20 September 2016</td>
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<tr>
<td>Revision received</td>
<td>21 September 2016</td>
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<tr>
<td>Accepted</td>
<td>22 September 2016</td>
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</tbody>
</table>

Editor: Dr. Andrea Leibfried

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 02 June 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees, whose comments are shown below.

As you will see, the referees appreciate your analyses. However, substantial additional work is required to better support your conclusions and to broaden the significance of your findings. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers.

Importantly,
- please recapitulate the observed effects in human organoids (see reports from all three referees)
- the WAEs and the E4 receptor localization need to be better characterized, also in vivo.
- the use of small intestinal cells is not a good system to model colonic events, this issue thus needs to be addressed (see also reports from referee #2 and referee #3)

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as
soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

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Referee #1:

Miyoshi and colleagues studied mechanisms responsible for intestinal epithelial repair following a biopsy-induced injury. They conclude that wound-associated epithelial cells (WAE cells) are regulated by prostaglandin E2 and that the process is dependent on the Ptger4 receptor. The study is well done, well written, and of general interest. There are a few additional experiments that might improve the paper. These would confirm the relevance of the findings in humans and the in vitro results in whole animals:

1. Primary human epithelial cell lines should be propagated as spheroids. Differences in the sizes of the spheroids should be shown to occur in response to prostaglandins (agonists, antagonists), as in the mouse cells.

2. Mice are available that are deficient in the individual prostaglandin receptors. Using these mice it would be nice to show that the differentiation of WAE cells are dependent only on the presence of the Ptger4 and not any of the other receptors. Although the in vivo mice experiments in genetically modified mice (lacking the receptors) would make the conclusion of the paper stronger, they are not absolutely necessary to do for publication in the EMBO journal. The inclusion of the human cell lines, however, should be done to confirm the significance of the finding in people.

Minor comment: Fig.8 - it would be nice to indicate the EP4 receptors somewhere in the schematic drawing - since this is the point of the paper.

Referee #2:

The paper by Miyoshi et al. describes an EP4 controlled, non-canonical WNT dependent mechanism of wound repair. The authors used state-of-the-art techniques, which they developed at least in part by themselves. Overall the manuscript is well written, the figures are of high quality and the conclusions are mostly robust. Nevertheless, there are some points that need to be addressed to improve the strength of the manuscript and to clarify the conclusions the authors draw.

Points to be addressed:

1. In general the usage of in vitro cultures is a great opportunity to study differentiation and stem cell phenotypes in an efficient manner, as described by the authors. The manuscript would benefit massively from data on human organoids. Therefore, some major experiments should be reproduced with human tissue derived organoids. This would give a direct translation into human diseases and would enhance the overall conclusion of the paper.

2. In addition the question remains whether the use of small intestinal cells is the best comparison to the used in vivo model. Or whether colonic organoid cultures resemble a more relevant in vitro model? Could the authors repeat some of their work in colonic organoids.

3. It's controversial to use a single marker to discriminate a cell type or state. Additional markers should be investigated to describe the population of WAEs more precisely. In addition, the authors show in Figure 2D the reduction of Cldn4 when using EP4i. Is Cldn4 regulated after EP1i-EP3i treatment? Additionally, the authors should describe the specificity of Cldn4 to WAEs, by analysing the expression of Cldn4 in the spheroids used in Figures 3E and 6I.
4. The authors state in the text that similar wound areas were generated for the data in Figure 7A and 7B. In this case, it would be more conclusive to express the data as relative to initial wound area.

5. The immunohistochemistry (IHC) quality of Figure EV4 is low and the conclusion drawn is not properly described. The increased cytoplasmic β-catenin appears at similar high levels in the most apical part, of the not wounded areas, in the crypts. Additionally, the immunofluorescence in Figure 7D is not conclusive as well. To clarify the high Wnt activity of in vivo WAEs the authors should analyse the expression of the already used Wnt targets Lrg5 and Axin2.

6. In line with point 5, the proliferative status of WAEs described in Figure 7D is of interest to recapitulate the conclusion from Figure 3E in vivo.

7. The Model in Figure 8 would increase in quality if the described mechanism is indicated.

Referee #3:

The paper attempts to clarify the initial response in the intestinal wound repair process by focusing on the mechanism of differentiation from intestinal stem/progenitor cells to wound associated epithelial (WAE) cells. The authors show that PGE2-Ptger4 pathway is a key pathway in regulating the formation of WAE cells during wound repair.

**Major comments**

1) If I understand it correctly, the stem cell/progenitor cell-enriched spheroids are forced to differentiate via growth factor withdrawal in the presence/absence of PGE2 and the resulting effect on lineage specification is then examined using markers. It is unclear to me why the jejunum was used exclusively to generate these spheroids when the in vivo wounding experiments are conducted in the colon. Are the findings relevant for all areas of the intestinal tract? Does it depend on the nature of the wound (shallow biopsies versus deep biopsies for instance)? What is the relevance of the findings for chronic injury conditions in the gut - is PGE2 signaling impaired in the presence of chronic inflammation for example? Is it conserved in humans? Can and should be easily tested using human spheroids/organoids.

2) What is the role of the stem cells in the PGE2-driven WAE response? Where is the E4 receptor expressed in the small intestinal/colonic crypts - is it on stem cells or selected progenitor compartments in the TA compartment? If, as surmised, PGE2 signaling is skewing a default enterocyte differentiation pathway from stem/progenitor cells, then it would be useful to accurately define the cells expressing the E4 receptor in vivo (via in situ or IHC). Is enterocyte differentiation blocked in favor of WAE lineage specification in vivo following PGE2 treatment? This could be directly evaluated using Lgr5-driven lineage tracing models (also in vitro, using regular 3D organoids which faithfully recapitulate the endogenous stem cell-driven epithelial renewal process).

3) I would like to see better validation of the expression profiling data using IHC/in-situ to more accurately define the temporal changes occurring in the spheroids following modulation of the Prostaglandin signaling. Is there any direct effect on stem cell identity or non-enterocyte lineage specification (also in vivo)?

**Minor comments**

1) In spheroid culturing experiments, no information for the number of spheroids proceeded to the differentiation medium culture is given. Was it same number in all conditions because if not, it is unfair to compare spheroid areas?

2) Page8 Line8 Lacking a figure number. Probably ‘Figure 3B’ instead of ‘Figure 3’.

3) Page8 Line26 It is not clear to me that PGE2 treated cells are ultrastructurally differentiated. The PGE2 treated cell in Figure 3C looks more similar to stem cells and even so different from in vivo WAE cell showed in Figure 3E. Could you give some statistical data (i.e. N/C ratio or brush border length) to make it easy to understand?
4) Page 11 Line 6: To conclude that 'PGE2 not only augments the WAE program of differentiation but also suppresses the enterocyte program of differentiation', additional markers to Fabp1 must be used (also for other lineages).

5) Figure 6A, B: Although the protein level of nuclear β-catenin in EP4i treated cells is low, that of cytoplasm is also low. Is it able to fully explain the low level of nuclear protein by only the inhibition of GSK-3β, or is there also a decrease of overall β-catenin expression?

1st Revision - authors' response 01 September 216

Point-By-Point Response to Referee Suggestions

Referee #1:

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1. Primary human epithelial cell lines should be propagated as spheroids. Differences in the sizes of the spheroids should be shown to occur in response to prostaglandins (agonists, antagonists), as in the mouse cells.

Response: All three reviewers made this point and we agree that the inclusion of human spheroid data would benefit this manuscript and extend the significance of these findings in mouse intestinal epithelial cells. We evaluated three human ileal spheroid lines to reproduce central experiments that we performed using mouse intestinal epithelial cells. We show that human spheroids displayed a morphological change and mRNA induction of multiple WAE markers in response to dmPGE2 treatment, similar to mouse cells. The dmPGE2 response in human spheroids was also mediated by EP4, and inhibition of EP4 with pharmacological inhibitors promoted enterocyte differentiation shown by two markers. Thus, the regulation of WAE cell formation through PGE2-EP4 signaling appears to be conserved between mouse and human intestinal epithelial cells.

Action taken: Representative images and quantitative data for human spheroid size and Cldn4 mRNA are included in Figure 2. Additional markers are shown in Figure EV2. The text has been modified to reflect the addition of the human epithelial cell data in the Materials and Methods section (Pg. 21). Results section (included below) and Discussion section (included below). Results Pg. 7: "We then tested whether PGE2 signaling through EP4 was important for the formation of WAE cells in human lines. In response to treatment with dmPGE2, human ileal spheroids exhibited morphological changes and induction of CLDN4 mRNA (Figure 2J-L) similar to mouse spheroids. EP4i blocked these responses (Figure 2J-L). Thus, PGE2-EP4 signaling appears to be a conserved mechanism in mouse and human."

Results Pg. 9: "We validated the genes diffuse panbronchiolitis critical region 1 (Dpcr1) and CD55 decay accelerating factor for complement B (Cd55b; also known as Daf2) as novel mRNA markers for WAE cells that were induced by PGE2 signaling through EP4 receptor in mouse and human intestinal epithelial cells (Figure 4E, F and Figure EV2)."

Results Pg. 10: "Therefore, we analyzed the genes most highly enriched after EP4i treatment and found many genes known to be highly enriched in enterocytes, including Fabp1, Ace2, and Maoa (Gordon, Elshourbagy et al., 1985, Hamming, Timens et al., 2004, Iemhoff & Hulsmann, 1971, Narisawa, Hoylaerts et al., 2007, Sivasubramaniam, Finch et al., 2003). We used quantitative PCR to validate the enrichment of these mRNAs in the EP4i-treated spheroids (mouse and human) (Figure 5B and Figure EV2) and immunostaining to validate the expression of Ace2 protein (Figure 5C) in the EP4i-treated spheroids."
Discussion Pg. 16: "Here, we identified PGE\textsubscript{2} as a potent inductive factor that directly acts on crypt epithelial cells to produce this atypical epithelial cell type. This mechanism appears to be conserved in mouse and human."

2. Mice are available that are deficient in the individual prostaglandin receptors. Using these mice it would be nice to show that the differentiation of WAE cells is dependent only on the presence of the Ptg4 and not any of the other receptors. Although the in vivo mice experiments in genetically modified mice (lacking the receptors) would make the conclusion of the paper stronger, they are not absolutely necessary to do for publication in the EMBO journal. The inclusion of the human cell lines, however, should be done to confirm the significance of the finding in people.

Response: We agree with the reviewer that in vivo assessment of the contribution of the other prostaglandin receptors using mouse models would strengthen the conclusion of the paper. We explored the possibility of obtaining Ptg1, Ptg2 and Ptg3 genetically modified mice. However, Ptg1-deficient mice were not commercially available and Ptg2-deficient mice and Ptg3 conditional mutant (flox) mice required cryo-recovery. As prostaglandins are known to affect the responses of multiple cell types involved in colonic wound healing, it would be important to generate mice that lacked these receptors specifically in the intestinal epithelium to definitively confirm that EP1-3 are not directly required for WAE cell formation. Thus, we would not able to complete the suggested experiment within the 90-day time frame provided for revision.

Action taken: None.

Minor comment: Fig.8 - it would be nice to indicate the EP4 receptors somewhere in the schematic drawing - since this is the point of the paper.

Response: We thank the reviewer for this suggestion. We have clarified this point in our model schematic.

Action taken: A representation of the EP4 receptor has been included Figure 9 (formerly Figure 8).

Referee #2:

The paper by Miyoshi et al. describes an EP4 controlled, non-canonical WNT dependent, mechanism of wound repair. The authors used state-of-the-art techniques, which they developed at least in part by themselves. Overall the manuscript is well written, the figures are of high quality and the conclusions are mostly robust. Nevertheless, there are some points that need to be addressed to improve the strength of the manuscript and to clarify the conclusions the authors draw. Points to be addressed:

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Response: We respectfully point out that in the first version of the manuscript, we had partially addressed this issue. In an attempt to bridge in vivo colonic WAE cells with in vitro small intestinal WAE cells, we performed a global comparison between the transcriptional profiles generated from in vitro small intestinal WAE cells to laser capture procured in vivo colonic WAE cells. There was a significant overlap of the WAE-enriched genes from each data set and similar pathway enrichment (Figure 4), suggesting that there is a link between these two models. We have clarified this in the text and made a separate paragraph for this point.

In addition, we added validation of key representative markers using colonic epithelial spheroids to further bridge these models. We think the small intestinal data is important because it shows the potential to generate these cells throughout the intestine.

Action taken: We included validation data for key representative markers in colon spheroids cultured as stem cells or in differentiation medium with dmPGE$_2$ or EP4i in Figure EV2. The text has been modified to reflect the addition of these data in the Results section (included below).

Results Pg. 8-9: "To confirm that in vitro WAE cells were transcriptionally similar to in vivo WAE cells, we compared the gene sets from Clusters 5 and 6 to previous microarray data obtained from laser capture microdissected WAE cells that covered colonic biopsy wounds (Miyoshi et al., 2012). We found a statistically significant overlap of the in vivo and in vitro WAE cell gene sets (p=0.0026) by hypergeometric probability test and similar pathway over-representation (Figure 4C). One difference between these two data sets was that the in vivo WAE cell cluster was additionally enriched for genes associated with cytokine and chemokine signaling pathways, which was likely a consequence of the inflammatory response that occurred in the wound bed. These data suggest that small intestinal WAE cells generated in vitro have similarity to colonic WAE cells in vivo."

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Results Pg. 10: "For mouse colonic spheroids, we validated that EP4i treatment resulted in induction of a colonocyte marker, Car4 (Kaiko, Ryu et al., 2016) (Figure EV2)."
3. It's controversial to use a single marker to discriminate a cell type or state. Additional markers should be investigated to describe the population of WAEs more precisely. In addition, the authors show in Figure 2D the reduction of Cldn4 when using EP4i. Is Cldn4 regulated after EP1i-EP3i treatment? Additionally, the authors should describe the specificity of Cldn4 to WAEs, by analysing the expression of Cldn4 in the spheroids used in Figures 3E and 6I.

Response: In previous publications, we utilized Cldn4 protein expression as a WAE marker (see Seno et al., PNAS 2009), which we have now confirmed in spheroids. While Cldn4 mRNA distinguishes WAE cells and enterocytes in vitro, we also observed Cldn4 mRNA expression in our in vitro stem cell cultures. Therefore we used our transcriptional profiling data to identify additional WAE mRNA markers, as requested by the reviewer. We found that Dpcr1 and Cd55b were reliable mRNA markers for WAE cells. We tested the effects of EP1, EP2, EP3 and EP4 inhibition on the mRNA expression of Cldn4 as well as Dpcr1 and Cd55b.

Action taken: Cldn4 immunostaining of spheroids has been added to Figure 4. Cldn4 mRNA expression data and high-power images of stem and dmPGE2-treated spheroids have been added to Figure EV1.

Quantitative data showing Cldn4 mRNA expression with EP1i-EP3i treatment has been added to Figure 2. Validation of the WAE mRNA markers Dpcr1 and Cd55b has been added to Figure 4 and Figure 6.

The text has been modified to reflect the addition of these data in the Materials and Methods section (Pg. 22) and in the Results section (included below).

Results Pg. 9: "Cellular markers to distinguish WAE cells are lacking. We previously reported Cldn4 protein expression in WAE cells in vivo (Seno et al., 2009). We confirmed this expression pattern in WAE cells generated in vitro (Figure 4D). Cldn4 mRNA robustly distinguishes dmPGE2- and EP4i-treated spheroids, but is also expressed in stem cell-enriched spheroids (Figure EV1). Despite this, mitotic state (Figure 3) and morphology (Figure EV1) can be used to distinguish stem and WAE spheroids. Thus, we used our transcriptional profiling data to identify additional mRNA markers that were enriched in dmPGE2-treated spheroids as compared to both stem and EP4i-treated spheroids. We validated the genes diffuse panbronchiolitis critical region 1 (Dpcr1) and CD55 decay accelerating factor for complement B (Cd55b; also known as Daf2) as novel mRNA markers for WAE cells that were induced by PGE2 signaling through EP4 receptor in mouse and human intestinal epithelial cells as well as mouse colonic epithelial cells (Figure 4E, F and Figure EV2)."

Results Pg. 11: "To do this, we first determined the time course of differentiation; mRNA expression levels of WAE cell genes (Dpcr1 and Cd55b) and enterocyte genes (Fabp1 and Ace2) were assessed at 2, 6, 12 and 24 hours after starting treatment with differentiation medium containing dmPGE2 or EP4i. The WAE cell mRNAs were detected by 6 hours of treatment (Figure 6A)."

Results Pg. 11: "If spheroids were treated during the second 12-hour period with dmPGE2, then the WAE cell markers Dpcr1 and Cd55b were expressed, regardless of the treatment during the initial 12 hours (Figure 6C)."

4. The authors state in the text that similar wound areas were generated for the data in Figure 7A and 7B. In this case, it would be more conclusive to express the data as relative to initial wound area.

Response: The major finding in wounded mice that are deficient for EP4 in the intestinal epithelium is that the wounds do not heal properly. The most dramatic demonstration of this phenotype is that there is a lack of covering of the wound by Cldn4-positive WAE cells and a substantial clot that is still adherent to the wound beds of these mice. To quantify this phenotype, we compared the surface area of the original wounds to the surface area of the remaining wound bed (i.e., percent healing), as requested by the reviewer. We have also measured the areas of the adherent clots.

Action taken: Quantitative data showing the percent healing (1 – [Day 4 wound area/original wound area] x 100) and the area of the fibrin clots has been added to Figure 8 (formerly Figure 7).
The text has been modified to reflect these changes in the Materials and Methods section (Pg. 20) and in the Results section (included below).

Results Pg. 15: "Although similar numbers of crypts were initially removed during biopsy injury, we consistently observed decreased healing in the Vil\textsuperscript{Cre} Ptger4\textsuperscript{flx/flx} mice compared to the controls four days post-injury by whole-mount microscopy (Figure 8A, B), suggesting impaired or delayed healing. In addition, fibrin clots were frequently found to be covering the wound surface in Vil\textsuperscript{Cre} Ptger4\textsuperscript{flx/flx} mice to a greater extent than control mice (Figure 8C)."

5. The immunohistochemistry (IHC) quality of Figure EV4 is low and the conclusion drawn is not properly described. The increased cytoplasmic β-catenin appears at similar high levels in the most apical part, of the not wounded areas, in the crypts. Additionally, the immunofluorescence in Figure 7D is not conclusive as well. To clarify the high Wnt activity of in vivo WAEs the authors should analyse the expression of the already used Wnt targets Lgr5 and Axin2.

Response: We have provided higher power images of WAE cells and colonocytes stained with β-catenin. These images show that we can detect nuclear β-catenin in WAE cells but not surface colonocytes. We have provided new single channel images for Cldn4 in Fig 8 (formerly Fig 7) to clarify the greatly diminished presence of WAE cells in EP4-deficient wounds. We have added H&E stains that more clearly show the phenotype and labeled areas of the images.

For the last point, we do not believe that WAE cells have high Wnt activity, just maintenance of nuclear beta-catenin (see porcupine results). We show in vitro that these cells do not express Lgr5 or Axin2 (canonical Wnt signaling readout) and that they are non-proliferative. To further support these conclusions in vivo, we have provided in situ hybridization for Axin2 in colonic wounds from control mice and wholemount images of biopsy wounds from Lgr5-GFP mice that show in vivo WAE cells are negative for these markers.

Action taken: Higher power images of β-catenin staining of in vivo WAE cells and colonocytes have been added to Figure EV5. Representative images for Axin2 in situ hybridization and Lgr5-GFP expression of the WAE cells covering biopsy wounds have also been added to Figure EV5. We have provided new Cldn4 immunofluorescence and H&E images of wound beds from control and EP4-deficient mice to Figure 8. The text has been modified to reflect the addition of these data in the Materials and Methods section (Pg. 20 & 22-23) and in the Results section (included below).

Results Pg. 15: "Histological analysis of wounded regions showed that a layer of flattened, Cldn4-positive cells was covering the wound surface in control injured mice (Figure 8D, E). These wound surface cells also exhibited accumulation of nuclear β-catenin (compared to surface epithelial cells of uninjured regions of colonic tissue), lacked Axin2 and Lgr5 expression, and were non-proliferative, similar to in vitro WAE cells (Figure EV5). In contrast, a layer of flattened, Cldn4-positive epithelial cells was lacking at the Vil\textsuperscript{Cre} Ptger4\textsuperscript{flx/flx} wound surfaces (Figure 8D, E). Instead, Cldn4-positive cells were only located at the edge of the wound and were frequently non-adherent. These data confirm that expression of EP4 in the intestinal epithelium is critical for complete covering of the wound bed with WAE cells and proper epithelial restitution in response to intestinal biopsy injury."

6. In line with point 5, the proliferative status of WAEs described in Figure 7D is of interest to recapitulate the conclusion from Figure 3E in vivo.

Response: We agree with the reviewer. We have previously published data from wild type mice showing that WAE cells are not labeled with BrdU and are not Ki67-positive (Seno et al., PNAS 2009). We have confirmed this in our current studies using Vil-Cre negative; EP4\textsuperscript{flx/flx} mice. The limited number of epithelial cells that are associated with the edge of wounds in injured Vil-Cre positive; EP4\textsuperscript{flx/flx} mice are also not proliferative. We also added additional data using an EdU incorporation assay to support the non-proliferative status of in vitro WAE cells.

Action taken: We have added representative Ki67 staining of colonic biopsy wounds to Figure EV5 and EdU detection in spheroids to Figure 3. The text has been modified in the Materials and Methods (Pg. 22-23) and in the Results section (included below) to reflect these additions.
Results Pg. 8: "We validated that PGE₂- and EP₄i-treated cells were post-mitotic by assessing proliferation with an EdU incorporation assay (Figure 3E, F) and a spheroid proliferation reporter line (Cdc25a-CRBLuc; Figure 3G) (Sun et al., 2015)."

Results Pg. 15: "These wound surface cells also exhibited accumulation of nuclear β-catenin (compared to surface epithelial cells of uninjured regions of colonic tissue), lacked Axin2 and Lgr5 expression, and were non-proliferative, similar to in vitro WAE cells (Figure EV5)."

7. The Model in Figure 8 would increase in quality if the described mechanism is indicated.

Response: We agree with the reviewer.

Action taken: We have edited the schematic in Figure 8 to include a description of the mechanism.

Referee #3:

The paper attempts to clarify the initial response in the intestinal wound repair process by focusing on the mechanism of differentiation from intestinal stem/progenitor cells to wound associated epithelial (WAE) cells. The authors show that PGE2-Ptger4 pathway is a key pathway in regulating the formation of WAE cells during wound repair.

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Results Pg. 8-9: "To confirm that in vitro WAE cells were transcriptionally similar to in vivo WAE cells, we compared the gene sets from Clusters 5 and 6 to previous microarray data obtained from laser capture microdissected WAE cells that covered colonic biopsy wounds (Miyoshi et al., 2012). We found a statistically significant overlap of the in vivo and in vitro WAE cell gene sets (p=0.0026) by hypergeometric probability test and similar pathway over-representation (Figure 4C). One difference between these two data sets was that the in vivo WAE cell cluster was additionally enriched for genes associated with cytokine and chemokine signaling pathways, which was likely a consequence of the inflammatory response that occurred in the wound bed. These data suggest that small intestinal WAE cells generated in vitro have similarity to colonic WAE cells in vivo."

Results Pg. 9: "We validated the genes diffuse panbronchiolitis critical region 1 (Dpcr1) and CD55 decay accelerating factor for complement B (Cd55b; also known as Daf2) as novel mRNA markers for WAE cells that were induced by PGE₂ signaling through EP₄ receptor in mouse and human small intestinal epithelial cells as well as mouse colonic epithelial cells (Figure 4E, F and Figure EV2)."
Results Pg. 10: "For mouse colonic spheroids, we validated that EP4i treatment resulted in induction of a colonocyte marker, Car4 (Kaiko, Ryu et al., 2016) (Figure EV2)."

1B) Does it depend on the nature of the wound (shallow biopsies versus deep biopsies for instance)?

Response: We have found that superficial biopsy injuries (i.e., that which leaves the crypt base) are healed within a few hours. The source of these cells is the crypt base progenitors.

Action taken: None.

1C) What is the relevance of the findings for chronic injury conditions in the gut - is PGE2 signaling impaired in the presence of chronic inflammation for example?

Response: This is an interesting question. However, numerous additional experiments would be required to answer this question and to determine how one or combinations of inflammatory cytokines may or may not alter the effects of PGE2 on WAE differentiation. This is beyond the scope of this current manuscript.

Action taken: None.

1D) Is it conserved in humans? Can and should be easily tested using human spheroids/organoids.

Response: All three reviewers made this point and we agree that the inclusion of human spheroid data would benefit this manuscript and extend the significance of these findings in mouse intestinal epithelial cells. We evaluated three human ileal spheroid lines to reproduce central experiments that we performed using mouse intestinal epithelial cells. We show that human spheroids displayed a morphological change and mRNA induction of multiple WAE markers in response to dmPGE2 treatment, similar to mouse cells. The dmPGE2 response in human spheroids was also mediated by EP4, and inhibition of EP4 with pharmacological inhibitors promoted enterocyte differentiation shown by two markers. Thus, the regulation of WAE cell formation through PGE2-EP4 signaling appears to be conserved between mouse and human intestinal epithelial cells.

Action taken: Representative images and quantitative data for human spheroid size and Cltn4 mRNA are included in Figure 2. Additional markers are shown in Figure EV2. The text has been modified to reflect the addition of the human epithelial cell data in the Materials and Methods section (Pg. 21), Results section (included below) and Discussion section (included below).

Results Pg. 7: "We then tested whether PGE2 signaling through EP4 was important for the formation of WAE cells in human lines. In response to treatment with dmPGE2, human ileal spheroids exhibited morphological changes and induction of CLDN4 mRNA (Figure 2J-L) similar to mouse spheroids. EP4i blocked these responses (Figure 2J-L). Thus, PGE2-EP4 signaling appears to be a conserved mechanism in mouse and human."

Results Pg 9: "We validated the genes diffuse panbronchiolitis critical region 1 (Dpcr1) and CD55 decay accelerating factor for complement B (Cd55b; also known as Daf2) as novel mRNA markers for WAE cells that were induced by PGE2 signaling through EP4 receptor in mouse and human intestinal epithelial cells (Figure 4E, F and Figure EV2)."

Results Pg 10: "Therefore, we analyzed the genes most highly enriched after EP4i treatment and found many genes known to be highly enriched in enterocytes, including Fabp1, Ace2, and Maoa (Gordon, Elshourbagy et al., 1985, Hamming, Timens et al., 2004, Lemhoff & Hulsmann, 1971, Narisawa, Hoylaerts et al., 2007, Sivasubramaniam, Finch et al., 2003). We used quantitative PCR to validate the enrichment of these mRNAs in the EP4i-treated spheroids (mouse and human) (Figure 5B and Figure EV2) and immunostaining to validate the expression of Ace2 protein (Figure 5C) in the EP4i-treated spheroids."

Discussion Pg. 16: "Here, we identified PGE2 as a potent inductive factor that directly acts on crypt epithelial cells to produce this atypical epithelial cell type. This mechanism appears to be conserved in mouse and human."

2A) What is the role of the stem cells in the PGE2-driven WAE response?
Response: We have previously shown that WAE cells emanate from stem cells in crypts adjacent to the wound (Miyoshi et al., Science 2012). Thus, stem cells are the source of WAE cells in vivo. As associations between PGE\(_2\) and stemness have been reported in colon cancer cells, we tested if PGE\(_2\) affected stem cell activity in vitro. We used Cdc25-luciferase spheroids (Sun et al., Cell Host & Microbe 2015), spheroid size and mRNA expression of Lgr5 and MKi67 to investigate whether dmPGE\(_2\) could alter proliferation of spheroids enriched for Lgr5-positive stem cells. We found no evidence for an effect of dmPGE\(_2\) on stem cell proliferation.

**Action taken:** We added these data to Appendix File S2 and altered the text to reflect these additions in the Results section (included below).

**Results Pg. 8:** "We also found that dmPGE\(_2\) treatment had no obvious effects on the proliferation of stem cell spheroids (Appendix File S2)."

2B) Where is the EP4 receptor expressed in the small intestinal/colonic crypts - is it on stem cells or selected progenitor compartments in the TA compartment? If, as surmised, PGE2 signaling is skewing a default enterocyte differentiation pathway from stem/progenitor cells, then it would be useful to accurately define the cells expressing the E4 receptor in vivo (via in situ or IHC).

Response: We confirmed previous studies that show Ptger4 mRNA is expressed in the intestinal epithelium by in situ hybridization (Morimoto et al. Am J Physiol GLP 1997). All epithelial cells in the small intestine and colon appear to express Ptger4 mRNA, with enrichment at the tips of villi in the small intestine. This was also supported by expression data obtained from RNAs procured by laser capture micro-dissection of specific intestinal epithelial compartments (Miyoshi et al., Science 2012). There are no commercial antibodies against EP4 that we found gave specific staining (using EP4-deficient spheroids as an important negative control).

**Action taken:** We have added our Ptger4 in situ hybridization results to Appendix Figure S1. We have modified the text in the Materials and Methods section (Pg. 23) and the Results section (included below) to reflect the addition of these data.

**Results Pg. 6:** "These data corroborated a previous in situ hybridization study reporting mRNA expression of Ptger1 and Ptger4 throughout the intestinal epithelium (Morimoto, Sugimoto et al., 1997) and our own validation of Ptger4 mRNA localization (Appendix Figure S1)."

2C) Is enterocyte differentiation blocked in favor of WAE lineage specification in vivo following PGE2 treatment? This could be directly evaluated using Lgr5-driven lineage tracing models (also in vitro, using regular 3D organoids which faithfully recapitulate the endogenous stem cell-driven epithelial renewal process).

Response: WAE differentiation requires injury (cell loss) and PGE\(_2\) in vivo, as exogenous treatment of PGE\(_2\) in non-wounded areas does not convert enterocytes into WAE cells morphologically. The proposed lineage tracing experiment does not extend our knowledge beyond the BrdU tracing (Seno et al., PNAS 2009) and the Villin-Cre tracing experiments (Miyoshi et al., Science 2012) that we have previously performed. These experiments clearly showed that WAE cells emanate from the stem and progenitor cells in adjacent crypts. We have also shown that WAE cells are replaced by enterocytes when PGE\(_2\) is diminished in the wound during later repair (Manieri et al., Gastro 2012).

**Action taken:** None

3) I would like to see better validation of the expression profiling data using IHC/in-situ to more accurately define the temporal changes occurring in the spheroids following modulation of the Prostaglandin signaling. Is there any direct effect on stem cell identity or non-enterocyte lineage specification (also in vivo)?

Response: We agree with the reviewer that additional validation of the gene expression data and definition of the temporal changes occurring in spheroids in response to dmPGE\(_2\) would benefit the manuscript. We identified and validated additional mRNA markers of WAE cells using the array data. We have defined the induction of these markers over time (testing in a time course from 6 - 24 hrs post induction), which will be useful for other investigators. We have also performed immunostaining for WAE and enterocyte markers. In addition, we investigated potential effects of
PGE2 on non-enterocyte lineages and found that this factor most dramatically affects the enterocyte lineage. We evaluated expression of goblet, endocrine and Paneth cell markers in dmPGE2- and EP4i-treated spheroids and found modest differences in mRNA abundance for these markers. There were no obvious changes in lineage allocation in uninjured areas of dmPGE2-treated mice (data not shown).

**Action taken:** Additional mRNA and protein markers for WAE cells and enterocytes have been added to Figures 4 and 5, respectively. Temporal data for these additional mRNA markers have added to Figure 6. Analysis of non-enterocyte lineages has been added to Figure EV3. The text has been modified to reflect these additions in the Materials and Methods section (Pg. 22) and in the Results section (included below).

**Results Pg 9:** "Cellular markers to distinguish WAE cells are lacking. We previously reported Cldn4 protein expression in WAE cells in vivo (Seno et al., 2009). We confirmed this expression pattern in WAE cells generated in vitro (Figure 4D). Cldn4 mRNA robustly distinguishes dmPGE2- and EP4i-treated spheroids, but is also expressed in stem cell-enriched spheroids (Figure EV1). Despite this, the spheroids' mitotic state (Figure 3) and morphology (Figure EV1) can be used to distinguish stem and WAE spheroids. Thus, we used our transcriptional profiling data to identify additional mRNA markers that were enriched in dmPGE2-treated spheroids as compared to both stem and EP4i-treated spheroids. We validated the genes diffuse panbronchiolitis critical region 1 (Dper1) and CD55 decay accelerating factor for complement B (Cd55b; also known as Daf2) as novel mRNA markers for WAE cells that were induced by PGE2 signaling through EP4 receptor in mouse and human intestinal epithelial cells (Figure 4E, F and Figure EV2)."

**Results Pg 10:** "Therefore, we analyzed the genes most highly enriched after EP4i treatment and found many genes known to be highly enriched in enterocytes, including Fabp1, Ace2, and Maaa (Gordon, Elshourbagy et al., 1985, Hamming, Timens et al., 2004, Iemhoff & Hulsmann, 1971, Narisawa, Hoylaerts et al., 2007, Sivasubramaniam, Finch et al., 2003). We used quantitative PCR to validate the enrichment of these mRNAs in the EP4i-treated spheroids (mouse and human) (Figure 5B and Figure EV2) and immunostaining to validate the expression of Ace2 protein (Figure 5C) in the EP4i-treated spheroids."

**Results Pg. 10-11:** "We also assessed secretory cells (endocrine, goblet and Paneth cells) in the EP4i-treated spheroids and found that these constituted a very small proportion (~5%) of the total cell number (Figure EV3). Overall, the transcriptional analysis and its validation suggested that PGE2-EP4 signaling regulates a cell fate decision primarily between enterocytes and WAE cells."

**Results Pg. 11:** "To do this, we first determined the time course of differentiation; mRNA expression levels of WAE cell genes (Dper1 and Cd55b) and enterocyte genes (Fabp1 and Ace2) were assessed at 2, 6, 12 and 24 hours after starting treatment with differentiation medium containing dmPGE2 or EP4i. The WAE cell mRNAs were detected by 6 hours of treatment (Figure 6A). In contrast, enterocyte mRNA induction did not occur until 12 to 24 hours of treatment (Figure 6B)."

**Minor comments**

1) In spheroid culturing experiments, no information for the number of spheroids proceeded to the differentiation medium culture is given. Was it same number in all conditions because if not, it is unfair to compare spheroid areas?

**Response:** We agree with the reviewer that additional information on our method for measuring spheroid areas should be provided. For comparisons within a single line of a particular genotype, the same spheroid-Matrigel mixture was plated into multiple wells that were subsequently provided with stem, WAE or enterocyte media. Thus, the three media comparison groups were always seeded at the same density. For comparisons between lines with different genotypes, the spheroids would be plated at a very similar density and treated as described above. The areas of individual spheroids were measured for a large number of spheroids from multiple experiments and multiple wells to produce the most robust data possible. To control for potential differences in the initial size of the epithelial cell clusters immediately after passaging (as there can be some small differences in the number of cells in each "cluster" of epithelial cells that will reform a new spheroid from passage to passage), we normalized the dmPGE2- and EP4i-treated spheroid areas to the stem cell areas from the same passage.
Action taken: The text has been edited in the Materials and Methods section to clarify this point. Materials and Methods Pg. 22: "ImageJ (Schneider et al., 2012) was used to determine the average individual spheroid area from bright-field images taken in the center of each tissue culture plate well of each sample (n = 4 four images [i.e., wells] and a minimum of 50 spheroids analyzed per sample). Similar densities of spheroids were seeded for all area measurement experiments."

2) Page8 Line8: Lacking a figure number. Probably 'Figure 3B' instead of 'Figure 3'.
Response: We thank the reviewer for pointing out our omission.
Action taken: We have edited the indicated text.

3) Page8 Line26: It is not clear to me that PGE2 treated cells are ultrastructurally differentiated. The PGE2 treated cell in Figure 3C looks more similar to stem cells and even so different from in vivo WAE cell showed in Figure 3E. Could you give some statistical data (i.e. N/C ratio or brush border length) to make it easy to understand?
Response: We agree with the reviewer that the addition of quantitative data would help support our conclusions from the ultrastructural analysis. We measured the cytoplasmic:nuclear ratio and microvillar length, as suggested. We found that both WAE cells and enterocytes had increased cytoplasmic:nuclear ratio compared to stem cells. The average microvillar length of WAE cells was significantly greater than that of stem cells, but less than that of enterocytes. These quantitative data support our conclusion that the dmPGE2-treated cells have ultrastructural characteristics consistent with being differentiated intestinal epithelial cells.
Action taken: Quantitative data for the cytoplasmic:nuclear ratio and microvillar length has been included in Figure 4. The text has been modified to reflect the addition of this data in the Materials and Methods section (Pg. 25) and in the Results section (included below).

Results Pg. 9: "To further confirm that in vitro WAE cells resembled in vivo WAE cells, we next compared their ultrastructure and histology. Cells treated with dmPGE2 had an increased cytoplasmic to nuclear ratio compared to spheroid stem cells and an apical brush border (although the microvilli were short), consistent with being a differentiated intestinal epithelial cell type (Figure 4G-I)."

4) Page11 Line6: To conclude that 'PGE2 not only augments the WAE program of differentiation but also suppresses the enterocyte program of differentiation', additional markers to Fabp1 must be used (also for other lineages).
Response: We agree with the reviewer that the inclusion of additional markers for the enterocyte lineage would strengthen the conclusion of the manuscript. To this end, we have assessed additional enterocyte mRNA markers (Ace2, Maoa) in key experiments throughout the manuscript. We have also included immunofluorescence data showing that Ace2 protein is detected specifically in the EP4i-treated spheroids and enterocytes in vivo (and not in WAE cells). The investigation of other intestinal lineages was described above in our responses to Referee 3, Point 3.
Action taken: Quantitative data for additional enterocyte mRNA markers has been included in Figures 5 and 6. Immunofluorescence for the enterocyte marker Ace2 has been added to Figure 5. The text has been modified to reflect the addition of this data in the Materials and Methods section (Pg. 22) and in the Results section (included below).

Results Pg. 10: "Therefore, we analyzed the genes most highly enriched after EP4i treatment and found many genes known to be highly enriched in enterocytes, including Fabp1, Ace2, and Maoa (Gordon, Elshourbagy et al., 1985, Hamming, Timens et al., 2004, Lembhoff & Hulsmann, 1971, Narisawa, Hoylaerts et al., 2007, Sivasubramaniam, Finch et al., 2003). We used quantitative PCR to validate the enrichment of these mRNAs in the EP4i-treated spheroids (mouse and human) (Figure 5B and Figure EV2) and immunostaining to validate the expression of Ace2 protein (Figure 5C) in the EP4i-treated spheroids."

5) Figure 6A, B: Although the protein level of nuclear β-catenin in EP4i treated cells is low, that of
cytoplasm is also low. Is it able to fully explain the low level of nuclear protein by only the inhibition of GSK-3β, or is there also a decrease of overall β-catenin expression?

Response: We have data suggesting that β-catenin protein is being actively degraded in the EP4i-treated cells (i.e., phospho-S33/S37/T41 β-catenin is present in the nucleus of these cells). This is one possible explanation for the data that we see, but we cannot fully explain the phenomena at this time.

Action taken: None.

2nd Editorial Decision
20 September 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the referees again, whose comments are enclosed. As you will see, the referees appreciate the amendments and now support publication in The EMBO Journal. I am thus happy to accept your manuscript in principle for publication here.

REFEREE REPORTS

Referee #2: The authors have addressed all my concerns in the revision process. This is an excellent manuscript worthy of publication.

Referee #3: No further comment
Corresponding Author Name: Thaddeus S. Steppenback
Journal Submitted to: EMBO Journal
Manuscript Number: EMBO-2016-94660

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A. Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates
- if n>5, the individual data points from each experiment should be plotted and any statistical test employed should be justified

Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (e.g. cell line, species name)
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entities that are being measured
- an explicit mention of the biological and chemical entities that are altered/defined in a controlled manner
- the exact sample size (n) for each experimental group/condition, given as a number: not a range
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.)
- a statement of how many times the experiment shown was independently replicated in the laboratory
- definitions of statistical methods and measures:
  - common tests, such as t-test (specify whether paired or unpaired), simple t tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P-values = x but not P-values < x
  - definition of ‘center value’ as median or average
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

Please follow the guidelines set out in the authorship guidelines on Data Presentation.

B. Statistics and general methods

1. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

Sample size was determined by a pilot experiment that was used to statistically justify the sample size used in the manuscript.

2. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

We performed multiple independent experiments with mice born in different litters (each with littermate control and experimental mice) and multiple biopies (3-4) were performed in each animal.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

Animals were excluded from analysis only if they became ill or their weight dropped below 90% of their original weight at the start of the experiment.

2. Were any steps taken to minimize the effects of confounding bias when allocating animals/samples to treatment (e.g. randomization procedure)? If so, please describe.

All mice were allocated into groups based on genotype alone. All mice underwent the same procedure (spaying injury)

2. For animal studies, include a statement about randomization even if no randomization was used.

There was no need to randomize mice (see above)

2. Were any steps taken to minimize the effects of confounding bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If so, please describe.

For all pathologic analyses, we blinded all the samples to the person performing the data collection and analysis.

2. For animal studies, include a statement about blinding even if no blinding was done.

All data collection and analysis for animal experiments was blinded.

2. For every figure, are statistical tests justified as appropriate?

Yes.

3. For the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.

Yes, we used Prism software to test for normal distribution.

3. Were there estimations of variance within each group of data?

Yes, we included error bars on all groups in all experiments.

3. Was variance similar between the groups that are being statistically compared?

Yes.

C. Reagents

This information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

1. Are the reagents and assays used for the experiments described in sufficient detail to allow other laboratories to reproduce the results?

Yes.

2. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

We performed multiple independent experiments with mice born in different litters (each with littermate control and experimental mice) and multiple biopies (3-4) were performed in each animal.

2. Were any steps taken to minimize the effects of confounding bias when allocating animals/samples to treatment (e.g. randomization procedure)? If so, please describe.

All mice were allocated into groups based on genotype alone. All mice underwent the same procedure (spaying injury)

2. For animal studies, include a statement about randomization even if no randomization was used.

There was no need to randomize mice (see above)

2. Were any steps taken to minimize the effects of confounding bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If so, please describe.

For all pathologic analyses, we blinded all the samples to the person performing the data collection and analysis.

2. For animal studies, include a statement about blinding even if no blinding was done.

All data collection and analysis for animal experiments was blinded.

2. For every figure, are statistical tests justified as appropriate?

Yes.

3. For the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.

Yes, we used Prism software to test for normal distribution.

3. Were there estimations of variance within each group of data?

Yes, we included error bars on all groups in all experiments.

3. Was variance similar between the groups that are being statistically compared?

Yes.
D- Animal Models

5. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile (e.g., Antibodypedia, see link at top right for a list of selected agents and toxins (APHIS/CDC) (see link at top right). According to our biosecurity guidelines, provide a statement only if it could.

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

8. For all hyperlinks, please see the table at the top right of the document.

9. For experiments involving the use of animals, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

10. We recommend consulting the ARRIVE guidelines (Lincoln et al., 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. See also: NIH and IACUC protocols (see link list at top right) and MRC ‘Guidelines for the use of experimental animals’ recommendations. Please confirm compliance.

11. We have deposited the transcriptomic profiling data generated as part of this study at Dryad (accession: DRYAD-552).

12. We have used primary cultures (mycoplasma-free) and u-WNB cell line (generated in-house) and 2D8 cells (acquired from ATCC).

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (Schulz et al., 2010) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.

15. For case report or prototype studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (Schulz et al., 2010) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.

E- Human Subjects

1. Identify the committee(s) approving the study protocol.

2. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principal set-out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

3. For publication of patient photos, include a statement confirming that consent to publish was obtained.

4. Report any restrictions on the availability (and/or) use of human data or samples.

5. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

6. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (Schulz et al., 2010) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.

7. For case report or prototype studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

F- Data Accessibility

8. Please provide accession codes for deposited data. See author guidelines, under ‘Data Deposit’.

9. Data deposition in a public repository is mandatory for:
   a. Protein, DNA and RNA sequences
   b. Microarray data structures
   c. Crystallographic data for small molecules
   d. Functional genomic data
   e. Proteomics and molecular interactions.

10. For human clinical and genomic datasets that should be published with few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement written in the study, such data should be deposited in one of the major public success controlled repositories such as GSEAF (access to gene set analysis or GSA (access to gene set analysis).

11. Insofar possible, primary and preprocessed data should be formally stored in a Data Availability section. Please state whether you have included this section.

   Examples:
   Primary Data
   Reference Data
   AF-Mn analysis of human histone deacetylase interactions in CEN-T cells (2010). PDB: P0D0D025B

12. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardization software (BioJava, Cytoscape) or standard sets of scripts (e.g., MATLAB). Authors are strongly encouraged to follow the REMARK guidelines (see link at top right) and deposit their model in a public database such as Biomodels (see link at top right) or JCB (see link at top right). If a computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.

G- Dual use research of concern

13. Confirm your study falls under dual use research or controls? Please follow biosecurity guidelines.

14. For all hyperlinks, please see the table at the top right of the document.