nature portfolio

Peer Review File

A tunable human intestinal organoid system achieves controlled balance between self-renewal and differentiation

Corresponding Author: Professor Xiaolei Yin

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Yang et al. established a novel protocol for human intestinal organoid culture that allows us to manipulate the capacity of self-renewal and differentiation of intestinal stem cells. Furthermore, they found the three small molecules cocktail (TpC) that increases the LGR5+ stem cell. This discovery is fantastic because the TpC-condition medium increased organoid-forming efficiency from bulk singlets to approximately 30% (Fig1g). That is terrific efficiency, which I have never seen because it means that the organoid-forming rate is practically 100%. Their single-cell RNA seq data shows that the percentage of stem cells (LGR5 high/low) in the organoids cultured under the TpC medium is about 30% (Fig2b).
(e.g.) Starting 1000 bulk cells containing 30% stem cells = 300 stem cells. If "ALL" of 300 stem cells can generate organoids, we will see 300 organoids. Then, the result of organoid formation efficiency becomes 30%.
Therefore, this manuscript's proposed condition medium of human intestinal organoids will significantly impact stem cell biology (organoid) regenerative medicine and drug discovery research. Consequently, I believe this paper contains sufficient interest and originality for publication. However, there are some conceptual and experimental concerns.

Major comments:
1. The authors developed an optimized culture condition with a combination of small molecules that balance self-renewal and differentiation. Compared with the previously conditioned medium (ES, IF, or IL22) for human intestinal organoid, the authors claim that the culture method in this manuscript is better at mimicking intestinal tissue condition and have the potential to generate a diverse range of intestinal cell types. Their human small intestinal organoids can be cultured while including many Paneth cells with high efficiency, but what about M cells under this medium condition? Organoids cultured in IF condition medium as a control contain the M cell population.

2. Fig. 1b: In this study, the authors originally targeted to generate LGR5-mNeonGreen organoid using CRISPR/Cas9. Therefore, they need to confirm this targeting strategy works well by Southern blotting and PCR-based genotyping analysis using the primers to recognize the transgene knocked in properly.

3. Fig.1d-j: The morphology of organoids cultured in TpC medium is heterogeneous (Second from the right in upper in Fig. 1d). According to my experiments, there are differences in cell populations just because of the different shapes of organoids, especially between the round-shaped and budding-structured ones. It is necessary to show honestly the immunohistochemistry data in both cases of round and budding structures of organoids.

4. Fig.1e-i: These are comparative experimental results of the TpC medium and the previously reported medium to demonstrate the superiority of the medium they developed in this manuscript. However, I wonder why they did not perform comparative experiments with the IF medium because their medium was based on IF medium (TpC = IF medium condition + TSA, pVc and CP673451). The parallel comparison in three conditional mediums (IL, TpC, and IF) will better validate the results.

5. Fig.1g: As I mentioned above, organoid-forming efficiency by this method is surprisingly high. Therefore, stem cell culture efficiency should be measured more accurately by classical methods. As the authors have LGR5-mNeonGreen organoid, they could collect the pure population of LGR5+ cells by FACS sorting. Testing organoid-forming assay from sorted singlets of LGR5+ stem cells will help demonstrate the medium's excellence.

6. Fig.2: They analyzed the population of Paneth cells in sc-RNA seq data using DEFENSIN and PRSS2. How about the expression pattern of LYSOZYME, the most typical marker gene for Paneth cells in mice and humans?

7. Fig.3a,b: To analyze the function of those three small molecules, the authors tested the effect of stemness and the ability to differentiate organoids by excluding factors one by one. When each factor, pVc or CP, was removed from the complete TpC medium (Those condition mediums contained CHIR+TSA+CP (-pVc) or CHIR+TSA+pVc (-CP), respectively), the number of stem cells decreased, as shown here. However, this result confused me greatly because this represented data is the opposite compared with Extended Data Fig.3 and the previous report (ref. 22, Yin et al. Nat Methods). The authors and Yin et al. demonstrated that the number of Lgr5+stem cells was increased in the organoids cultured in the presence of CHIR (Wnt agonist) and histone deacetylase inhibitors (VPA, TSA). The authors need to explain this discrepancy.

Furthermore, I also could not follow why the organoid growth was inhibited in the culture condition -CP because the medium still contains Wnt agonist, EGF, RSPO, BMP inhibitor, A83-01, IGF-1, FGF-2 that is almost the same as IF medium. Does TSA or pVc alone affect organoid growth?

8. Fig. 5m: The authors mentioned that Goblet cell differentiation was partially observed from the lineage of early enterocyte cells (shown in Result p13, lines 24-26). But which data supports this conclusion? It might help prove their idea if they could introduce the pure population of early enterocyte status and force into Goblet cell differentiation by inhibiting Notch signaling.

Minor Comments:
1. Fig.1c: The original ES medium (Sato et al. Gastroenterology) does not contain PGE2.

2. p12, line 15-16: As there are several reports previously that treatment DAPT in human intestinal organoids led to cell destiny towards secretory cells, please cite those papers.

3. p13, line 4-6: Same as above, please cite the previous report that the Hans Clevers group have already demonstrated that inhibition of the cell cycle using Gefenitib induces enteroendocrine cell differentiation (Basak et al. Cell Stem Cell 2017).

4. Methods (p16, line 16): There is no information about the approval number of this ethics.

5. Methods (p17, line 22): Please put a space between the number and the unit in the part of EGF and IGF.

6. Sup Table 2: The information of that sequence might be unclear. They should put more information appropriately. Which part is for the homology arm for the LGR5 locus?

Reviewer #2

(Remarks to the Author)
In the presented manuscript, Yang et al. provide a medium composition for the culture of adult stem cell-derived small intestinal organoids. Compared to other published media, the study presents a medium with higher secretory cell diversity and homeostatic stemness through the combination of previously described compounds and addition of three small molecules. Based on this medium, the authors explore how small intestinal cell types can be enriched through combinatorial inhibition/activation of certain pathways. While the manuscript clearly presents a push towards the secretory lineage, the downsides of this approach are not clearly enough addressed or at least in depth discussed in the current version. This includes the very low abundance of absorptive cell types, missing crypt-villus gradients of proliferation and cell-types (e.g. higher proliferation in the crypt), as well as phenotypic variability within cultures. While the manuscript presents intriguing findings and usable enrichment strategies of various cell types, it requires further mechanistic insights, addressing potential sources of noise, and especially proper comparisons to in vivo data to fully elucidate the cellular and molecular processes at play. Addressing these major and minor points will significantly enhance the scientific rigor and clarity of the study.

Major Points:
• Lack of Mechanistic Insight: The manuscript lacks mechanistic insight into the observed phenomena. Specifically, the manuscript mentions the use of HDAC inhibitors and their effects on chromatin rearrangements. It is important to investigate in general how these inhibitors affect the chromatin (over time). In more detail, one could try to resolve whether these inhibitors specifically target certain chromatin complexes (e.g., NuRD, SWI/SNF, PRC1/2) and how these effects relate to the de-differentiation and differentiation of intestinal stem cells. Overall, the potential role of HDAC inhibitors in maintaining cells in a plastic/regenerative state should be explored.
• There seems to be a high discrepancy between cell type abundances in the scRNAseq (Figure 2) and imaging (Figure 1). For example, LYZ1+ cells are shown in very high abundance throughout the whole organoid in Figure 1, while only 1% of all cells are counted as Paneth cells in Figure 2. Could the authors comment on this discrepancy?
• The UMAP representation of OLFM4 and ASCL2 expression appears inconsistent with their known roles as master transcriptional regulators. Especially OLFM4 shows widespread patterns. The authors should explain whether these genes are still functioning as regulators and provide details on when (day of organoid development) the single-cell RNA sequencing experiment was conducted.
• To enhance the significance of their medium composition, the authors should compare their results to published in vivo datasets to demonstrate the relevance and applicability of their findings.
• To clarify the impact of iBET inhibitors and TSA, perform additional sequencing experiments on LGR5-high, medium, low,

and negative cells. This could help identify the specific cell types and processes affected by these treatments.
• As an effect of the extensive inhibitors used in the presented manuscript, it likely destroys any intrinsic crypt-villus gradient as e.g. ISC and paneth cells seem to be rather randomly distributed throughout the organoid (e.g. Figure 1i,m). This should be clearly noted in the manuscript as a disadvantage of this protocol. Currently, at page 6 lines 2-6 it reads as “high degree of homogeneity”, which sounds positive.

Minor Points:
• Inconsistent Organoid Sizes: The manuscript displays different organoid sizes in various figures. It would be beneficial to provide a representative scheme explaining why different sizes were used and how they relate to the experiments. Figure 1 shows notably larger organoids than Figure 4, and the authors should address the morphological differences observed.
• Phenotypic Heterogeneity: There seems to be large heterogeneity during culture, as captured for example in brightfield images of Figure 1d. Can the authors argue on the source of this heterogeneity and how their phenotypes are different from each other in terms of marker expression?
• The used ALPI staining makes it very hard to estimate how many enterocytes there really are in mature organoids compared to the secretory lineage (Figure 1 I,j,l. The enterocyte fraction is counted to be only 16.1% according to scRNAseq, which is low compared to the estimate in vivo abundance of ~70% (e.g., Wang et al., 2019). The bias towards secretory cells should be clearly noted in the manuscript.
• Page 5, Line 21/22: The arrow does not point towards a double positive cell in Figure 1l as far as we can judge.
• The authors show that a positive LGR5 cell can grow out to form a fully developed organoid. Is this true for other cell types as well?
• The authors note that Figure 2g is only the secretory subset of the scRNAseq data. Why does it have EC, TA’s and ISC’s?
• Can the authors argue on the mechanism of transdifferentiation observed from EC to GC? Is it caused by the medium, which may partially block EC differentiation? Chromatin landscape (see major point).
• Figure 2n is hard to read correctly. I would suggest to show piecharts as secretory % of the whole data set, instead of showing the same data which is underneath as a barchart.
• Figure 3a/b indicates similar area but reduced LGR5 counts. This seems slightly contradicting. Can it be that the OLFM4 stem cell state is instead increased?
• In many metrics (Figure 3), +pVc.CP seems almost equally good to TpC. Where is the advantage of TpC over it?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my concerns, but this manuscript will not include some critical data (shown in the rebuttal letter). It is up to the editors to decide whether to revise the manuscript a second time in light of the data in rebuttal.
1. Rebuttal Fig.6: The authors demonstrated that LGR5-negative cells formed organoids more efficiently than LGR5-high ones in the TpC-conditioned medium. In response to this observation, they discussed how the TpC condition medium introduces the highly dynamic cell fate changes and plasticity of intestinal epithelial cells. It is very intriguing data, but unfortunately, the study suffers from major technical and an experimental design flaw. They need to show organoids can formed from LGR5+cells only using IF medium as a positive control. However, there might have never been any reports that non-stem cells can form organoids ex vivo, and I have no experience with that. There are some reports to identify robust stem cell markers in the human intestine and confirmed by performing organoid formation assay, LGR5 (Ishikawa et al. Gastroenterology 163(5), 2022), PTK7 (Jung et al. Stem Cell Rep 5(6), 2015), and EPHB2 (Jung et al. Nature Medicine 17(10), 2011). Those papers represented that human intestinal organoids are formed from only stem cell-positive cells. Therefore, they should explain the molecular mechanism of how organoids can be established from non-stem cells only when the TpC medium is used.
2. Rebuttal Fig.6: The authors discuss that the TpC medium introduces organoid formation derived from stem cell negative cells because it is a medium that promotes cell plasticity. However, Rebuttal Fig.5g indicates that organoid incubated in the TpC medium has more differentiated cells than in other mediums, which means the TpC medium is the medium that can efficiently induce differentiated cells. How do they explain this discrepancy?
3. The transdifferentiating experiment of Supplementary Fig.19 is problematic. From the general point of view of the organoid specialist, this short period is insufficient to induce differentiated cells, which means stem cells are still absolutely present in the organoids in the protocol. Therefore, it is possible that the MUC2-positive goblet cells observed at 1d+5d derived directly from stem cells but not enterocytes. The phenomenon of direct conversion of absorptive cells into secretory cells has not been reported not only in the intestine but also in other organs. Interpretation of scRNA-seq data alone is insufficient to explain this impactful biological event. Well-designed experiments should be used to prove it. The authors have the technology to make transgenic organoids that allow the isolate of only enterocytes (e.g., GFP knockin organoids in the ALPI gene locus by CRISPR/Cas9).
4. Response to my concern #7: I wondered why the stem cells decreased in this manuscript when the authors cultured organoid in +TSA (HDAC inhibitor) basal medium, which contains CHIR because a previous report indicated CHIR + VPA (another HDAC inhibitor) enhanced numbers of intestinal stem cell. In contrast, they claimed it is due to species specificity between mice and humans. However, their assertion is incorrect. The paper by Yin et al. (Nature Methods 2014, Supplementary Fig.3) represented that organoids are formed more efficiently in CHIR+VPA than in other conditions, not only in mice but also in humans.

Reviewer #2

(Remarks to the Author)
Dear Yang et al.,
Thank you for your comprehensive responses to our comments and your thoughtful revisions to the manuscript. We appreciate the effort you have put into addressing the concerns raised, and we believe that these changes have strengthened the work considerably. Below, we provide additional feedback based on your rebuttal and the revised manuscript.
Major points:
1. Chromatin-level analysis (e.g., ATAC-seq):
We understand that chromatin-level analysis was not within the original scope of your study. While we acknowledge your explanation, we believe that mapping the effects of the compounds on an epigenetic level would provide valuable insights into the mechanisms at play. This could be a fruitful avenue for future research, and we encourage you to consider such approaches in subsequent studies. Although it is not a current requirement, adding a brief mention of this as a limitation or a potential future direction in the discussion could strengthen the manuscript by outlining a clear path for future exploration.
2. Your rebuttal here is well-received. We are satisfied with your explanation and your recognition of other important studies in the field. The additional context you provided enriches the discussion and highlights your awareness of the broader landscape of research in this area.
3. References to human small Intestine only:
The focus on human small intestine markers is crucial for maintaining the manuscript's relevance and accuracy, and we are glad to see this adjustment was made. Please remove the references to colon-related studies, recent literature and dataset integrations show clear differences between colon and small intestine concerning the stem cell markers.
4. The corrections made to the labeling of EC cells and other textual refinements have enhanced the clarity of the manuscript. We commend your efforts in ensuring accuracy and clarity, as this will help prevent confusion in the field. Your proactive approach to refining the terminology and framework in this area is appreciated, and we believe the manuscript will contribute positively to ongoing research.
5. After reviewing the updated manuscript, we confirm that the revisions were carried out satisfactorily. The paper has benefitted from these changes and now reads more clearly and cohesively.
6. The final adjustments to the text are excellent. These have significantly improved the readability and scientific communication of your work, making it easier for other researchers in the field to follow your findings and conclusions.
General Questions:
Quantification of mNeonGreen intensity:
Could you clarify how mNeongreen signal has been quantified (e.g. per well or per segmented organoid)? And include it with the method section, please. We think it will enable future researchers to replicate your experiments accurately.
Cell density and Matrigel percentage:
For reproducibility purposes we would like to suggest emphasizing how many cells per microliter have been plated in a certain Matrigel percentage. Please include it in the methods section so that it stands out for future researchers looking to replicate your organoid culture conditions.
Conclusion:
Overall, we are very pleased with the revisions you have made in response to our comments. The manuscript is much improved and now provides a clearer contribution to the field, particularly regarding in vitro systems for the human small intestine. We believe that with these revisions, your work will be a valuable resource for future research, helping to advance the development of more physiologically relevant models.

We look forward to seeing this work published and believe it will be of great benefit to the scientific community.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I think the authors have answered the critiques satisfactorily, and your rebuttal comments here are well-received. But the one concern remains unresolved. Against my concern #2, the authors explained that the TpC medium simultaneously increases both the intestinal stem cell compartment and the number of differentiated cells by expanding the stem cell pool. Actually, they demonstrated that the “percentages” (not cell numbers) of intestinal stem cells, but also all lineage cells of the intestinal epithelium, were increased in Rebuttal Fig. 5. However, there must be a rise in some cell types and a fall in others if they analyzed the percentages of cell types that comprise the organoids. Why is there no cell type at all that is decreasing its proportion among the cells that make up the organoids? The authors should provide a clear explanation for that.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Yang et al. established a novel protocol for human intestinal organoid culture that allows us to manipulate the capacity of self-renewal and differentiation of intestinal stem cells. Furthermore, they found the three small molecules cocktail (TpC) that increases the LGR5+ stem cell. This discovery is fantastic because the TpC-condition medium increased organoid-forming efficiency from bulk singlets to approximately 30% (Fig1g). That is terrific efficiency, which I have never seen because it means that the organoid-forming rate is practically 100%. Their single-cell RNA seq data shows that the percentage of stem cells (LGR5 high/low) in the organoids cultured under the TpC medium is about 30% (Fig2b). (e.g.) Starting 1000 bulk cells containing 30% stem cells = 300 stem cells. If "ALL" of 300 stem cells can generate organoids, we will see 300 organoids. Then, the result of organoid formation efficiency becomes 30%.

Therefore, this manuscript's proposed condition medium of human intestinal organoids will significantly impact stem cell biology (organoid) regenerative medicine and drug discovery research. Consequently, I believe this paper contains sufficient interest and originality for publication. However, there are some conceptual and experimental concerns.

Major comments:

1. The authors developed an optimized culture condition with a combination of small molecules that balance self-renewal and differentiation. Compared with the previously conditioned medium (ES, IF, or IL22) for human intestinal organoid, the authors claim that the culture method in this manuscript is better at mimicking intestinal tissue condition and have the potential to generate a diverse range of intestinal cell types. Their human small intestinal organoids can be cultured while including many Paneth cells with high efficiency, but what about M cells under this medium condition? Organoids cultured in IF condition medium as a control contain the M cell population.

2. Fig. 1b: In this study, the authors originally targeted to generate LGR5-mNeonGreen organoid using CRISPR/Cas9. Therefore, they need to confirm this targeting strategy works well by Southern blotting and PCR-based genotyping analysis using the primers to recognize the transgene knocked in properly.

3. Fig.1d-j: The morphology of organoids cultured in TpC medium is heterogeneous (Second from the right in upper in Fig. 1d). According to my experiments, there are differences in cell populations just

because of the different shapes of organoids, especially between the round-shaped and budding-structured ones. It is necessary to show honestly the immunohistochemistry data in both cases of round and budding structures of organoids.

4. Fig.1e-i: These are comparative experimental results of the TpC medium and the previously reported medium to demonstrate the superiority of the medium they developed in this manuscript. However, I wonder why they did not perform comparative experiments with the IF medium because their medium was based on IF medium (TpC = IF medium condition + TSA, pVc and CP673451). The parallel comparison in three conditional mediums (IL, TpC, and IF) will better validate the results.

5. Fig.1g: As I mentioned above, organoid-forming efficiency by this method is surprisingly high. Therefore, stem cell culture efficiency should be measured more accurately by classical methods. As the authors have LGR5-mNeonGreen organoid, they could collect the pure population of LGR5+ cells by FACS sorting. Testing organoid-forming assay from sorted singlets of LGR5+ stem cells will help demonstrate the medium's excellence.

6. Fig.2: They analyzed the population of Paneth cells in sc-RNA seq data using DEFENSIN and PRSS2. How about the expression pattern of LYSOZYME, the most typical marker gene for Paneth cells in mice and humans?

7. Fig.3a, b: To analyze the function of those three small molecules, the authors tested the effect of stemness and the ability to differentiate organoids by excluding factors one by one. When each factor, pVc or CP, was removed from the complete TpC medium (Those condition mediums contained CHIR+TSA+CP (-pVc) or CHIR+TSA+pVc (-CP), respectively), the number of stem cells decreased, as shown here. However, this result confused me greatly because this represented data is the opposite compared with Extended Data Fig.3 and the previous report (ref. 22, Yin et al. Nat Methods). The authors and Yin et al. demonstrated that the number of Lgr5+stem cells was increased in the organoids cultured in the presence of CHIR (Wnt agonist) and histone deacetylase inhibitors (VPA, TSA). The authors need to explain this discrepancy.

Furthermore, I also could not follow why the organoid growth was inhibited in the culture condition -CP because the medium still contains Wnt agonist, EGF, RSPO, BMP inhibitor, A83-01, IGF-1, FGF-2 that is almost the same as IF medium. Does TSA or pVc alone affect organoid growth?

8. Fig. 5m: The authors mentioned that Goblet cell differentiation was partially observed from the lineage of early enterocyte cells (shown in Result p13, lines 24-26). But which data supports this conclusion? It might help prove their idea if they could introduce the pure population of early enterocyte status and force into Goblet cell differentiation by inhibiting Notch signaling.

Minor Comments:

1. Fig.1c: The original ES medium (Sato et al. Gastroenterology) does not contain PGE2.

2. p12, line 15-16: As there are several reports previously that treatment DAPT in human intestinal organoids led to cell destiny towards secretory cells, please cite those papers.

3. p13, line 4-6: Same as above, please cite the previous report that the Hans Clevers group have already demonstrated that inhibition of the cell cycle using Gefenitib induces enteroendocrine cell differentiation (Basak et al. Cell Stem Cell 2017).

4. Methods (p16, line 16): There is no information about the approval number of this ethics.

5. Methods (p17, line 22): Please put a space between the number and the unit in the part of EGF and IGF.

6. Sup Table 2: The information of that sequence might be unclear. They should put more information appropriately. Which part is for the homology arm for the LGR5 locus?

Reviewer #2 (Remarks to the Author):

In the presented manuscript, Yang et al. provide a medium composition for the culture of adult stem cell-derived small intestinal organoids. Compared to other published media, the study presents a medium with higher secretory cell diversity and homeostatic stemness through the combination of previously described compounds and addition of three small molecules. Based on this medium, the authors explore how small intestinal cell types can be enriched through combinatorial inhibition/activation of certain pathways. While the manuscript clearly presents a push towards the secretory lineage, the downsides of this approach are not clearly enough addressed or at least in depth discussed in the current version. This includes the very low abundance of absorptive cell types, missing crypt-villus gradients of proliferation and cell-types (e.g. higher proliferation in the crypt), as well as phenotypic variability within cultures. While the manuscript presents intriguing findings and usable enrichment strategies of various cell types, it requires further mechanistic insights, addressing potential sources of noise, and especially proper comparisons to in vivo data to fully elucidate the cellular and molecular processes at play. Addressing these major and minor points will significantly enhance the scientific rigor and clarity of the study.

Major Points:

• Lack of Mechanistic Insight: The manuscript lacks mechanistic insight into the observed phenomena. Specifically, the manuscript mentions the use of HDAC inhibitors and their effects on chromatin

rearrangements. It is important to investigate in general how these inhibitors affect the chromatin (over time). In more detail, one could try to resolve whether these inhibitors specifically target certain chromatin complexes (e.g., NuRD, SWI/SNF, PRC1/2) and how these effects relate to the de-differentiation and differentiation of intestinal stem cells. Overall, the potential role of HDAC inhibitors in maintaining cells in a plastic/regenerative state should be explored.

• There seems to be a high discrepancy between cell type abundances in the scRNAseq (Figure 2) and imaging (Figure 1). For example, LYZ1+ cells are shown in very high abundance throughout the whole organoid in Figure 1, while only 1% of all cells are counted as Paneth cells in Figure 2. Could the authors comment on this discrepancy?

• The UMAP representation of OLFM4 and ASCL2 expression appears inconsistent with their known roles as master transcriptional regulators. Especially OLFM4 shows widespread patterns. The authors should explain whether these genes are still functioning as regulators and provide details on when (day of organoid development) the single-cell RNA sequencing experiment was conducted.

• To enhance the significance of their medium composition, the authors should compare their results to published in vivo datasets to demonstrate the relevance and applicability of their findings.

• To clarify the impact of iBET inhibitors and TSA, perform additional sequencing experiments on LGR5-high, medium, low, and negative cells. This could help identify the specific cell types and processes affected by these treatments.

• As an effect of the extensive inhibitors used in the presented manuscript, it likely destroys any intrinsic crypt-villus gradient as e.g. ISC and paneth cells seem to be rather randomly distributed throughout the organoid (e.g. Figure 1i, m). This should be clearly noted in the manuscript as a disadvantage of this protocol. Currently, at page 6 lines 2-6 it reads as “high degree of homogeneity”, which sounds positive.

Minor Points:

• Inconsistent Organoid Sizes: The manuscript displays different organoid sizes in various figures. It would be beneficial to provide a representative scheme explaining why different sizes were used and how they relate to the experiments. Figure 1 shows notably larger organoids than Figure 4, and the authors should address the morphological differences observed.

• Phenotypic Heterogeneity: There seems to be large heterogeneity during culture, as captured for example in brightfield images of Figure 1d. Can the authors argue on the source of this heterogeneity and how their phenotypes are different from each other in terms of marker expression?

• The used ALPI staining makes it very hard to estimate how many enterocytes there really are in

mature organoids compared to the secretory lineage (Figure 1 i,j,l. The enterocyte fraction is counted to be only 16.1% according to scRNAseq, which is low compared to the estimate in vivo abundance of ~70% (e.g., Wang et al., 2019). The bias towards secretory cells should be clearly noted in the manuscript.

• Page 5, Line 21/22: The arrow does not point towards a double positive cell in Figure 1I as far as we can judge.

• The authors show that a positive LGR5 cell can grow out to form a fully developed organoid. Is this true for other cell types as well?

• The authors note that Figure 2g is only the secretory subset of the scRNAseq data. Why does it have EC, TA's and ISC's?

• Can the authors argue on the mechanism of transdifferentiation observed from EC to GC? Is it caused by the medium, which may partially block EC differentiation? Chromatin landscape (see major point).

• Figure 2n is hard to read correctly. I would suggest to show piecharts as secretory % of the whole data set, instead of showing the same data which is underneath as a barchart.

• Figure 3a/b indicates similar area but reduced LGR5 counts. This seems slightly contradicting. Can it be that the OLFM4 stem cell state is instead increased?

• In many metrics (Figure 3), +pVc.CP seems almost equally good to TpC. Where is the advantage of TpC over it?

Summary of the Revision:

We are grateful to the reviewers for their insightful comments and constructive suggestions for our manuscript. In response, we have conducted additional experiments, collected new data, and revised the text to clarify key points. Below, we provide a detailed, point-by-point response to address all the comments raised by the reviewers.

To facilitate the review process of our manuscript and rebuttal letter, we have included the new data as rebuttal figures in this letter, with references to the corresponding figures and text in the revised manuscript. Additionally, we have marked all changes in the revised manuscript with color highlighting.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Yang et al. established a novel protocol for human intestinal organoid culture that allows us to manipulate the capacity of self-renewal and differentiation of intestinal stem cells. Furthermore, they found the three small molecules cocktail (TpC) that increases the $LGR5^+$ stem cell. This discovery is fantastic because the TpC-condition medium increased organoid-forming efficiency from bulk singlets to approximately 30% (Fig1g). That is terrific efficiency, which I have never seen because it means that the organoid-forming rate is practically 100%. Their single-cell RNA seq data shows that the percentage of stem cells (LGR5 high/low) in the organoids cultured under the TpC medium is about 30% (Fig2b). (e.g.) Starting 1000 bulk cells containing 30% stem cells = 300 stem cells. If "ALL" of 300 stem cells can generate organoids, we will see 300 organoids. Then, the result of organoid formation efficiency becomes 30%.

Therefore, this manuscript's proposed condition medium of human intestinal organoids will significantly impact stem cell biology (organoid) regenerative medicine and drug discovery research. Consequently, I believe this paper contains sufficient interest and originality for publication. However, there are some conceptual and experimental concerns.

Thank you for the positive and encouraging feedback on our work and its implications for

stem cell biology, regenerative medicine, and drug discovery research. We have carefully considered your conceptual and experimental concerns and have revised our manuscript accordingly. Below, we address each comment in detail.

Major comments:

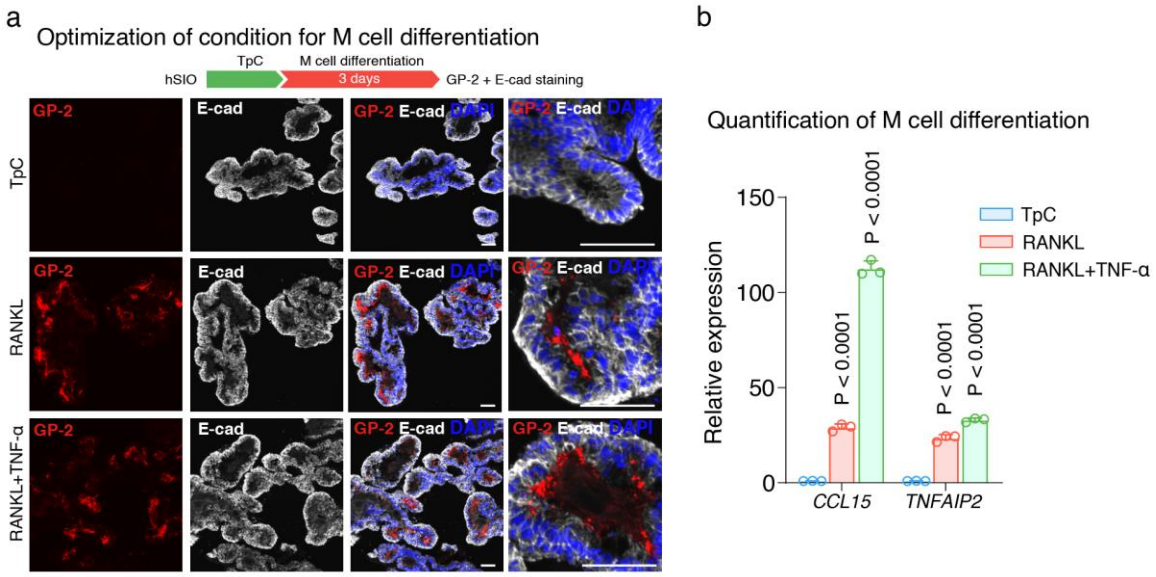
1. The authors developed an optimized culture condition with a combination of small molecules that balance self-renewal and differentiation. Compared with the previously conditioned medium (ES, IF, or IL22) for human intestinal organoid, the authors claim that the culture method in this manuscript is better at mimicking intestinal tissue condition and have the potential to generate a diverse range of intestinal cell types. Their human small intestinal organoids can be cultured while including many Paneth cells with high efficiency, but what about M cells under this medium condition? Organoids cultured in IF condition medium as a control contain the M cell population.

Response:

We appreciate your comment. To investigate the presence of M cells in our optimized organoid culture, we conducted an in-depth analysis of our sequencing data and performed immunostaining for the M cell marker GP-2. We did not find a significant GP-2^{+} M cell population in our optimized organoids (**Rebuttal Fig. 1; new Supplementary Fig. 18**). However, we performed additional experiments to clarify your question and summarize key findings:

1. M cells can be differentiated from TpC organoids with RANKL and TNF-α:

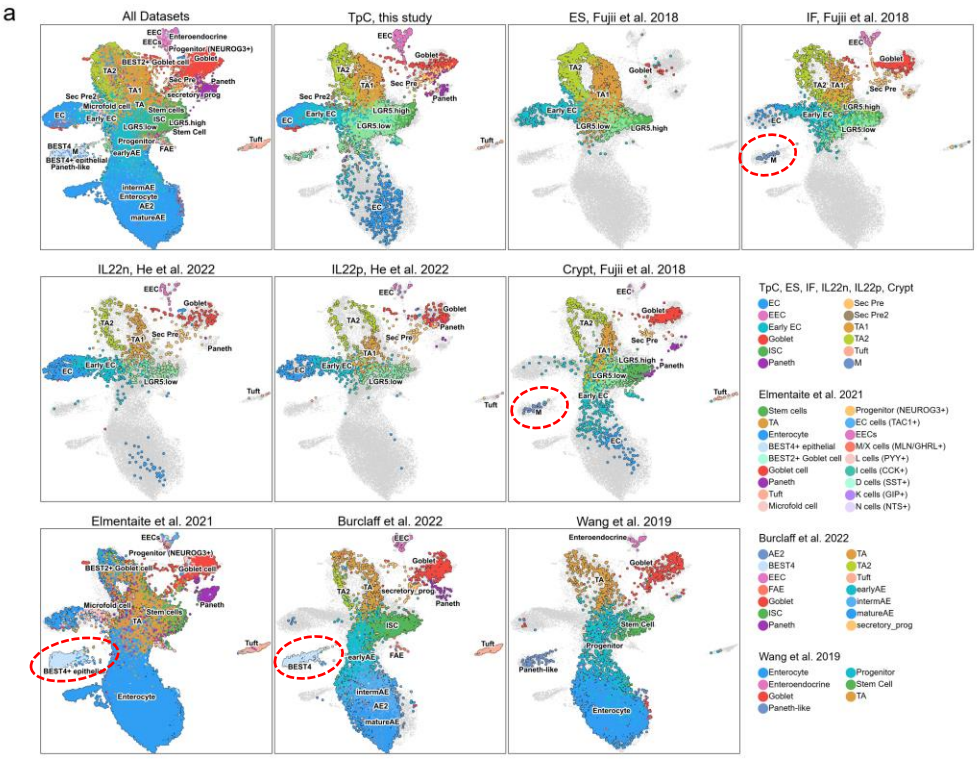
The cells in our TpC condition exhibit high plasticity. We performed direct M cell differentiation using conditions previously reported for mouse intestinal organoids[1,2]. We achieved high-efficiency differentiation of M cell in our human intestinal organoid system (**Rebuttal Fig. 1; new Supplementary Fig. 18**). These findings suggest that though M cells are rare in the optimized organoid model, the stem cells in this system have significant potential for multi-directional differentiation, including into M cells.

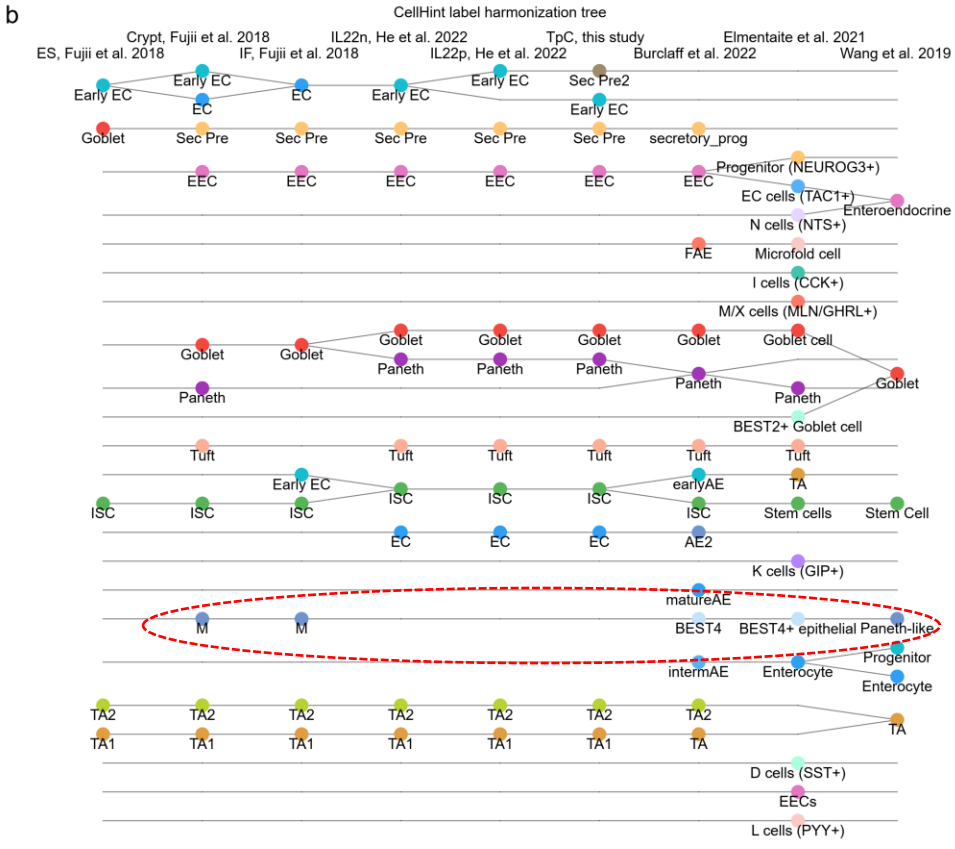


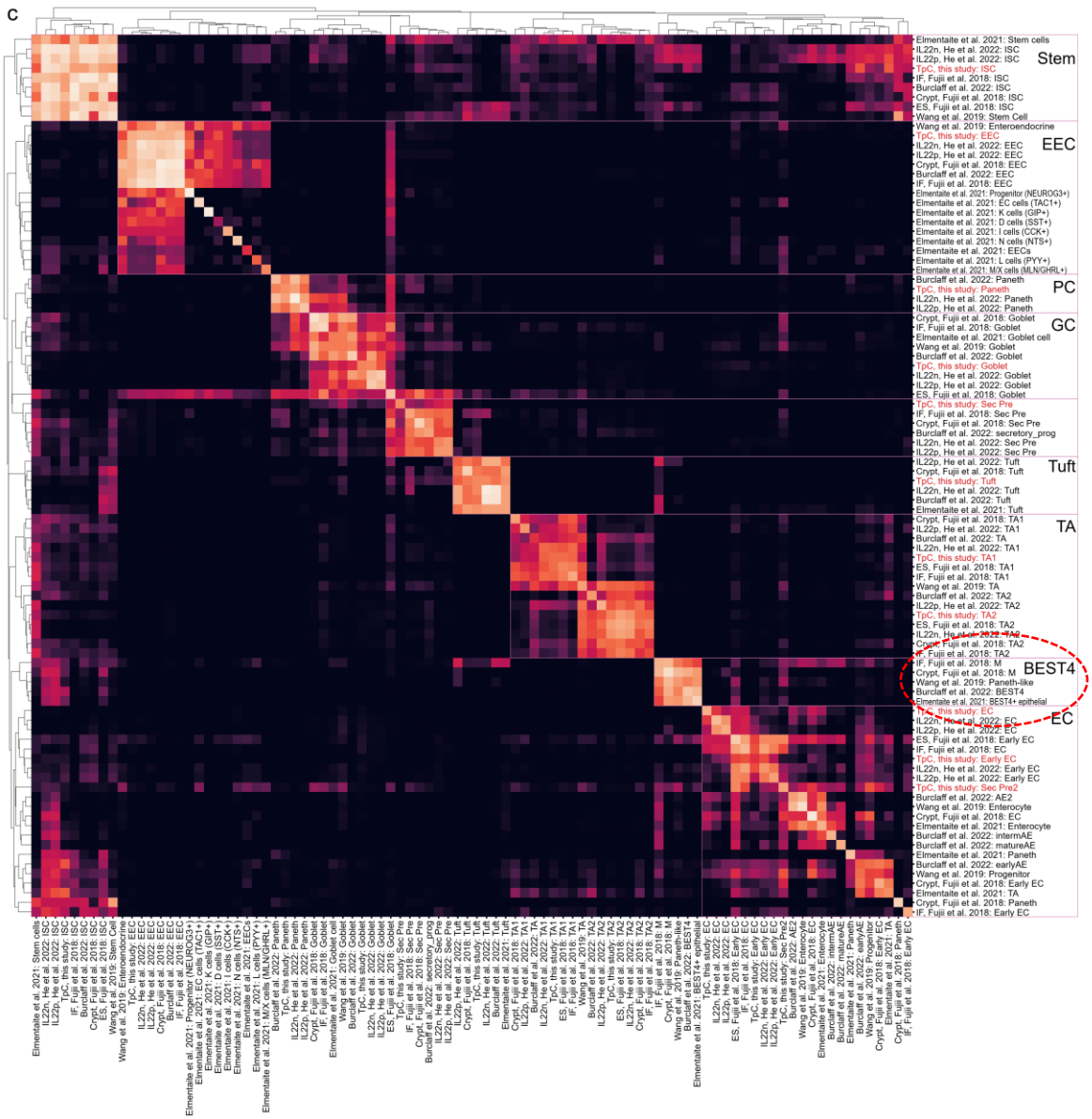
Rebuttal Fig.1 Induced M cell differentiation in hSIO

2. Previous identified M cells more closely resemble BEST4 cells.

While analyzing the M cell population in organoids, we found that previously reported M cells in the IF and Crypt datasets (Fujii et al., 2018) resemble BEST4 cells more closely, when compared with *in vivo* datasets (**Rebuttal Fig. 2; new Supplementary Fig. 6b and new Supplementary Fig. 7-9**).


a
All Datasets
TpC, this study
ES, Fujii et al. 2018
IF, Fujii et al. 2018
IL22n, He et al. 2022
IL22p, He et al. 2022
Crypt, Fujii et al. 2018
Elmentaite et al. 2021
Burclaff et al. 2022
Wang et al. 2019
TpC, ES, IF, IL22n, IL22p, Crypt
EC
EEC
Early EC
Goblet
ISC
Paneth
Sec Pre
Sec Pre2
TA1
TA2
Tuft
M
Elmentaite et al. 2021
Stem cells
TA
Enterocyte
BEST4+ epithelial
BEST2+ Goblet cell
Goblet cell
Paneth
Tuft
Microfold cell
Progenitor (NEUROG3+)
EC cells (TAC1+)
EECs
M/X cells (MLN/GHRL+)
L cells (PYY+)
I cells (CCK+)
D cells (SST+)
K cells (GIP+)
N cells (NTS+)
Burclaff et al. 2022
AE2
BEST4
EEC
FAE
Goblet
ISC
Paneth
TA
TA2
Tuft
earlyAE
intermAE
matureAE
secretory_prog
Wang et al. 2019
Enterocyte
Enteroendocrine
Goblet
Paneth-like
Progenitor
Stem Cell
TA


b
CellHint label harmonization tree
Crypt, Fujii et al. 2018
ES, Fujii et al. 2018
IF, Fujii et al. 2018
IL22n, He et al. 2022
IL22p, He et al. 2022
TpC, this study
Burclaff et al. 2022
Elmentaite et al. 2021
Wang et al. 2019
Early EC
EC
Sec Pre2
Goblet
Sec Pre
secretory_prog
EEC
Progenitor (NEUROG3+)
EC cells (TAC1+)
Enteroendocrine
N cells (NTS+)
FAE
Microfold cell
I cells (CCK+)
M/X cells (MLN/GHRL+)
Goblet cell
Paneth
BEST2+ Goblet cell
Tuft
earlyAE
TA
ISC
Stem cells
Stem Cell
AE2
K cells (GIP+)
matureAE
M
BEST4
BEST4+ epithelial Paneth-like
intermAE
Enterocyte
Progenitor
TA2
TA1
D cells (SST+)
EECs
L cells (PYY+)


c
Elmentaite et al. 2021: Stem cells
IL22n, He et al. 2022: ISC
IL22p, He et al. 2022: ISC
Stem
TpC, this study: ISC
IF, Fujii et al. 2018: ISC
Burclaff et al. 2022: ISC
Crypt, Fujii et al. 2018: ISC
ES, Fujii et al. 2018: ISC
Wang et al. 2019: Stem Cell
Wang et al. 2019: Enteroendocrine
TpC, this study: EEC
IL22n, He et al. 2022: EEC
IL22p, He et al. 2022: EEC
EEC
Crypt, Fujii et al. 2018: EEC
Burclaff et al. 2022: EEC
IF, Fujii et al. 2018: EEC
Elmentaite et al. 2021: Progenitor (NEUROG3+)
Elmentaite et al. 2021: EC cells (TAC1+)
Elmentaite et al. 2021: K cells (GIP+)
Elmentaite et al. 2021: D cells (SST+)
Elmentaite et al. 2021: I cells (CCK+)
Elmentaite et al. 2021: N cells (NTS+)
Elmentaite et al. 2021: EECs
Elmentaite et al. 2021: L cells (PYY+)
Elmentaite et al. 2021: M/X cells (MLN/GHRL+)
Burclaff et al. 2022: Paneth
TpC, this study: Paneth
IL22n, He et al. 2022: Paneth
IL22p, He et al. 2022: Paneth
PC
Crypt, Fujii et al. 2018: Goblet
IF, Fujii et al. 2018: Goblet
Elmentaite et al. 2021: Goblet cell
Wang et al. 2019: Goblet
Burclaff et al. 2022: Goblet
TpC, this study: Goblet
IL22n, He et al. 2022: Goblet
IL22p, He et al. 2022: Goblet
ES, Fujii et al. 2018: Goblet
GC
TpC, this study: Sec Pre
IF, Fujii et al. 2018: Sec Pre
Crypt, Fujii et al. 2018: Sec Pre
Burclaff et al. 2022: secretory_prog
IL22n, He et al. 2022: Sec Pre
IL22p, He et al. 2022: Sec Pre
IL22p, He et al. 2022: Tuft
Crypt, Fujii et al. 2018: Tuft
TpC, this study: Tuft
IL22n, He et al. 2022: Tuft
Burclaff et al. 2022: Tuft
Elmentaite et al. 2021: Tuft
Tuft
Crypt, Fujii et al. 2018: TA1
IL22p, He et al. 2022: TA1
Burclaff et al. 2022: TA
IL22n, He et al. 2022: TA1
TpC, this study: TA1
ES, Fujii et al. 2018: TA1
IF, Fujii et al. 2018: TA1
Wang et al. 2019: TA
Burclaff et al. 2022: TA2
IL22p, He et al. 2022: TA2
TpC, this study: TA2
ES, Fujii et al. 2018: TA2
IL22n, He et al. 2022: TA2
Crypt, Fujii et al. 2018: TA2
IF, Fujii et al. 2018: TA2
TA
IF, Fujii et al. 2018: M
Crypt, Fujii et al. 2018: M
Wang et al. 2019: Paneth-like
Burclaff et al. 2022: BEST4
Elmentaite et al. 2021: BEST4+ epithelial
BEST4
TpC, this study: EC
IL22n, He et al. 2022: EC
IL22p, He et al. 2022: EC
ES, Fujii et al. 2018: Early EC
IF, Fujii et al. 2018: EC
TpC, this study: Early EC
IL22n, He et al. 2022: Early EC
IL22p, He et al. 2022: Early EC
TpC, this study: Sec Pre2
Burclaff et al. 2022: AE2
Wang et al. 2019: Enterocyte
Crypt, Fujii et al. 2018: EC
Elmentaite et al. 2021: Enterocyte
Burclaff et al. 2022: intermAE
Burclaff et al. 2022: matureAE
Elmentaite et al. 2021: Paneth
Burclaff et al. 2022: earlyAE
Wang et al. 2019: Progenitor
Crypt, Fujii et al. 2018: Early EC
Elmentaite et al. 2021: TA
Crypt, Fujii et al. 2018: Paneth
IF, Fujii et al. 2018: Early EC
EC

Rebuttal Fig.2 Comparison of single-cell datasets of organoid and *in vivo*

References:

1. Knoop, K.A., *et al.* RANKL Is Necessary and Sufficient to Initiate Development of Antigen-Sampling M Cells in the Intestinal Epithelium. *J Immunol* **183**, 5738-5747 (2009).

2. Wood, M.B., Rios, D. & Williams, I.R. TNF-α augments RANKL-dependent intestinal M cell differentiation in enteroid cultures. *Am J Physiol-Cell Ph* **311**, C498-C507 (2016).

2. Fig. 1b: In this study, the authors originally targeted to generate LGR5-mNeonGreen organoid using CRISPR/Cas9. Therefore, they need to confirm this targeting strategy works well by Southern blotting and PCR-based genotyping analysis using the primers to recognize the transgene knocked in properly.

Response:

Thank you for this suggestion. To confirm the validity of our targeting strategy for generating LGR5-mNeonGreen organoids using CRISPR/Cas9, we performed genotyping and expression pattern analyses.

1. Direct genotyping analysis confirms correct genome editing:

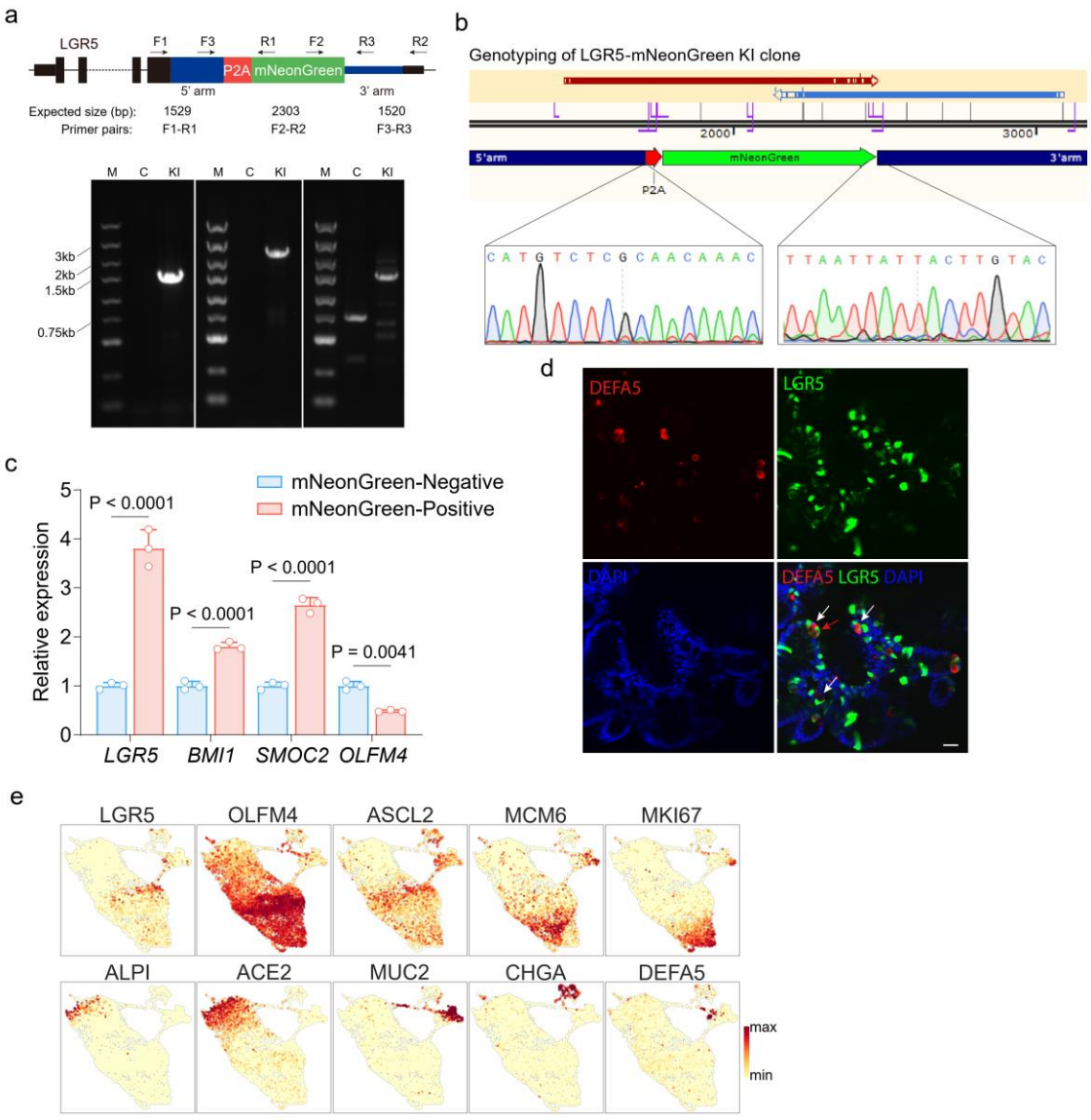
We designed primers to verify the integration of the mNeonGreen fragment into the target locus. PCR-based genotyping confirmed the correct insertion of mNeonGreen at the LGR5 locus, with gel electrophoresis showing the expected band sizes (**Rebuttal Fig. 3a; new Supplementary Fig. 1a**). Sequencing of the junction sites further validated precise genome editing (**Rebuttal Fig. 3b; new Supplementary Fig. 1b**).

2. Phenotype consistency of mNeonGreen and LGR5 expression pattern:

Due to technical challenges for Southern blotting, we instead sorted mNeonGreen-negative and -positive cells using FACS and assessed stemness markers by qPCR. The results showed high expression of LGR5 cell markers in mNeonGreen-positive cells, consistent with their expected expression pattern (**Rebuttal Fig. 3c; new Supplementary Fig. 1c**).

We also observed intermingled Paneth cells and LGR5-mNeonGreen cells in the organoids, consistent with their *in vivo* localization pattern (**Rebuttal Fig. 3d; new Fig. 1k**). The existence of LGR5-mNeonGreen/DEFA5 co-localized cells aligns with our single-cell sequencing results, where DEFA5-positive cells also express LGR5 (**Rebuttal Fig. 3e; Fig. 2d**), suggesting the expression pattern of mNeonGreen is consistent with LGR5.

These data indicate that the mNeonGreen reporter was correctly knocked into the target locus and functions well under our experimental conditions.



Rebuttal Fig.3 Confirmation of LGR5-mNeonGreen reporter system

3. Fig.1d-j: The morphology of organoids cultured in TpC medium is heterogeneous (Second from the right in upper in Fig. 1d). According to my experiments, there are differences in cell populations just because of the different shapes of organoids, especially between the round-shaped and budding-structured ones. It is necessary to show honestly the immunohistochemistry data in both cases of round and budding structures of organoids.

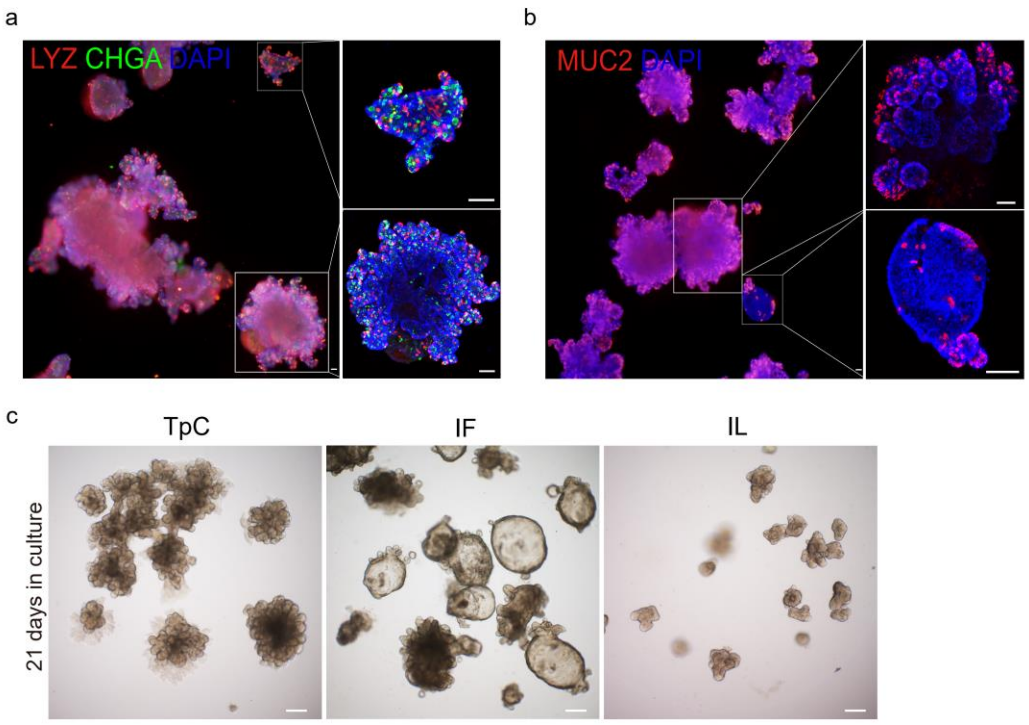
Response:

Thank you for this constructive comment. As suggested, we presented the full view and amplified images of both round-shaped and budding-structured organoids in the same field of immunofluorescence staining images (**Rebuttal Fig. 4a-b; new Supplementary Fig. 3d-e**). The representative images demonstrate that almost all organoids contain the primary secretory lineage cells—Paneth cells, goblet cells, and enteroendocrine cells (EEC)—regardless of whether they are round or budding. However, a small number of round-shaped organoids contained fewer differentiated cells compared to the budding ones.

From our single cell colony forming experiments (discussed in comment 5), we conclude that the observed heterogeneity in shape/structure, size, and cell composition primarily originates from the different types of cells that initiate the organoids starting from single cells. If the organoids are passaged as cell clumps, the heterogeneity in size and shape will be even larger, but this does not mean true heterogeneity in cell composition.

Organoids can be initiated by multiple cell types, including LGR5-negative cells (such as TA cells and early EC cells) and LGR5-positive cells. These different cell types have varying proliferation abilities, resulting in organoids of different sizes. Initially, all organoids appear as round colonies when growing from single cells (before day 10) and later form budding structures. Despite size differences, they ultimately achieve similar cell composition with high diversity, forming budding-structured organoids. Only a small number of organoids remain as round/cyst structures with lower cell diversity, likely due to the loss of stem cells (**Rebuttal Fig. 4a-b, new Supplementary Fig. 3d-e**). Therefore, organoids in the TpC condition are mostly homogeneous in terms of cell composition and diversity, regardless of their sizes or shapes.

Additionally, we observed that there are more budding-structured organoids cultured in TpC conditions than in IF conditions (**Rebuttal Fig. 4c; new Supplementary Fig. 2a**).



Rebuttal Fig. 4. Cell composition and structure in organoids

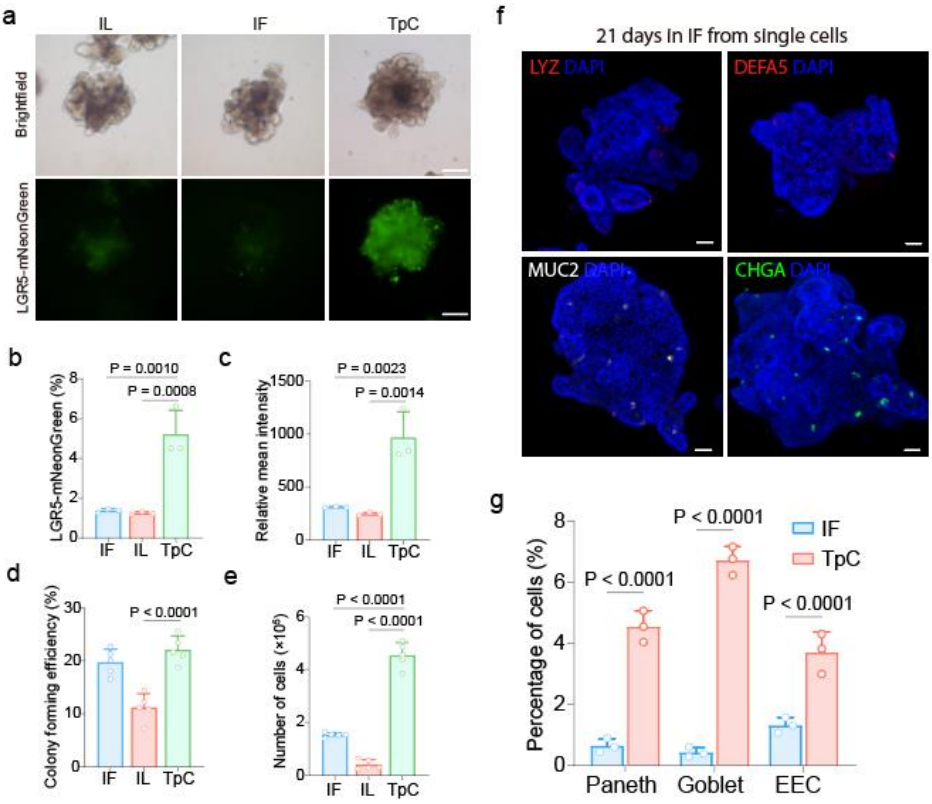
4. Fig.1e-i: These are comparative experimental results of the TpC medium and the previously reported medium to demonstrate the superiority of the medium they developed in this manuscript. However, I wonder why they did not perform comparative experiments with the IF medium because their medium was based on IF medium (TpC = IF medium condition + TSA, pVc and CP673451). The parallel comparison in three conditional mediums (IL, TpC, and IF) will better validate the results.

Response:

Thank you for the insightful comment. In light of this issue, we performed additional parallel comparisons and found the following: We compared the LGR5-mNeonGreen proportion and intensity among organoids cultured in IL, IF, and TpC conditions. Our results showed that TpC-organoids have the highest LGR5-stem cell percentage (**Rebuttal Fig. 5a-c; new Fig. 1d-f**). Organoids cultured in TpC-medium demonstrated the highest colony forming efficiency and proliferation ability, indicated by higher cell numbers (**Rebuttal Fig. 5d-e; new Fig. 1g-h**). We observed that TpC organoids contain a higher cellular diversity of Paneth cells (LYZ^+/$DEFA5^+$), goblet cells ($MUC2^+$), and enteroendocrine cells ($CHGA^+$) compared to those

in IF medium. This was quantified and shown in our results (**Rebuttal Fig. 5f-g; new Fig. 1i and new Supplementary Fig. 2b**).

These findings indicate that the TpC condition supports the generation of abundant secretory lineage cells without requiring an additional differentiation step, as is necessary with the IL condition.



Rebuttal Fig.5 Comparison of organoids cultured in IL, IF and TpC conditions

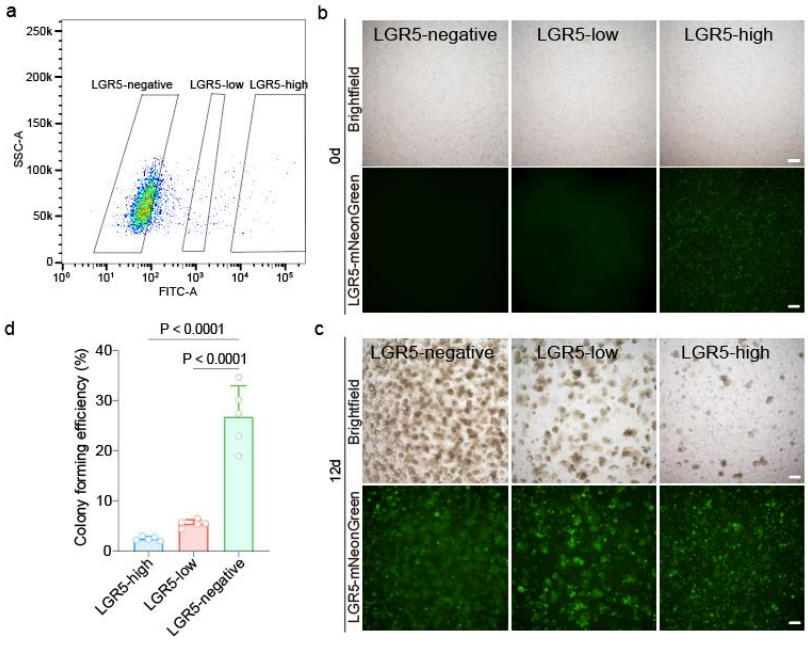
5. Fig.1g: As I mentioned above, organoid-forming efficiency by this method is surprisingly high. Therefore, stem cell culture efficiency should be measured more accurately by classical methods. As the authors have LGR5-mNeonGreen organoid, they could collect the pure population of LGR5+ cells by FACS sorting. Testing organoid-forming assay from sorted singlets of LGR5+ stem cells will help demonstrate the medium's excellence.

Response:

Thank you for the suggestion. As recommended, we sorted LGR5-high, LGR5-low and LGR5-

negative cells in TpC organoids by FACS and then seeding dissociated single cells in the same density, respectively (**Rebuttal Fig. 6a-b; new Supplementary Fig. 4a-b**). Interestingly, after 12 days growth, we found that the LGR5-high group possess lowest organoid-forming efficiency, while the LGR5-negative group contribute the majority of clone formation (**Rebuttal Fig. 6c-d; new Supplementary Fig. 4c-d**). Moreover, the organoids derived from LGR5-negative cells generated LGR5-mNeonGreen stem cells under TpC condition. These finding suggest that the organoids formation in TpC condition primarily originated from LGR5-negative cells rather than LGR5-stem cells, in consequence the organoid formation efficiency in TpC condition generally equal or exceed the percentage of LGR5 stem cells.

We believe the observed results is due to the highly dynamic cell fate changes and plasticity of intestinal epithelial cells. LGR5-high cells serve as stem cells capable of generating secretory cells, specifically Paneth cells. Additionally, non-LGR5 cells, including TA cells and partially differentiated EC cells, can also regenerate organoids under the TpC condition. This is consistent with recent findings in the mouse intestine, where TA cells or isthmus progenitor cells also have the capacity to act as stem cells and reconstitute intestine epithelial[1,2].



Rebuttal Fig.6 Organoid-forming assay of LGR5-high, low and negative cells

References:

1. Malagola, E., *et al.* Isthmus progenitor cells contribute to homeostatic cellular turnover and

support regeneration following intestinal injury. *Cell* **187**, 3056-3071 e3017 (2024).

2. Capdevila C., *et al*. Time-resolved fate mapping identifies the intestinal upper crypt zone as an origin of Lgr5+crypt base columnar cells. *Cell* **187**: (2024).

6. Fig.2: They analyzed the population of Paneth cells in sc-RNA seq data using DEFENSIN and PRSS2. How about the expression pattern of LYSOZYME, the most typical marker gene for Paneth cells in mice and humans?

Response:

Thank you for this question. We have accordingly provided the expression pattern of *LYZ*. We believe that *LYZ* is not a specific marker for Paneth cells at the mRNA level, but could serve as a specific marker at the protein level. Specifically:

1. *LYZ* is not a specific mRNA marker for Paneth cells:

Our data demonstrates that although *LYZ* mRNA does exhibit high expression in Paneth cell clusters, its expression is not limited to them. Notably, *LYZ* mRNA is detectable in diverse cell types including LGR5 stem cells, TA cells, early enterocytes (Early EC), and enterocytes (EC), suggesting that *LYZ* is not a specific marker for Paneth cells at the mRNA level (**Rebuttal Fig. 7**).

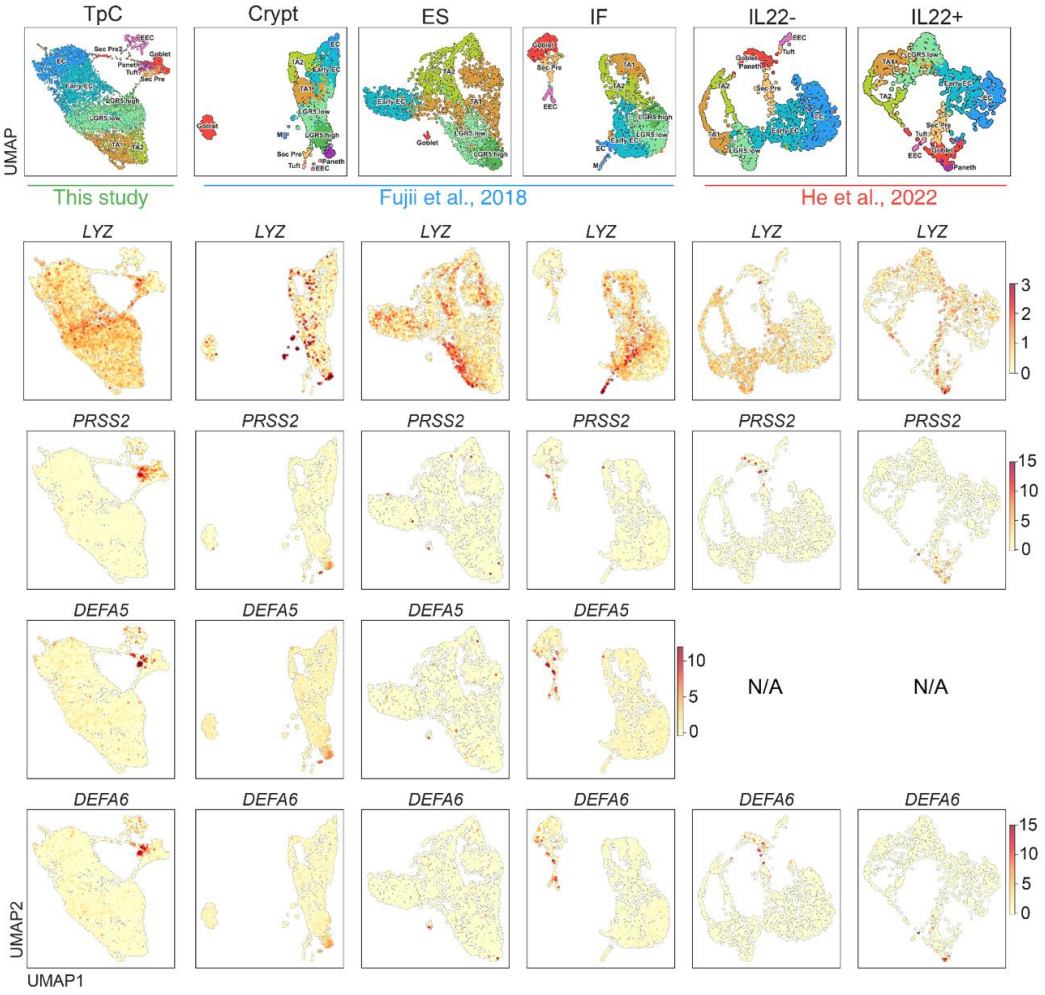
2. LYZ as a specific protein marker for Paneth cells:

We performed immunostaining of LYZ and DEFA5, another highly specific Paneth cell marker showing specific mRNA expression in single-cell RNA-seq data (**Rebuttal Fig.8; new Supplementary Fig. 3a-c**). Although LYZ and DEFA5 positive cells do not always co-localize, cells co-expressing both LYZ and DEFA5 constitute most Paneth cells. This suggests that LYZ could serve as a more specific marker at the protein level.

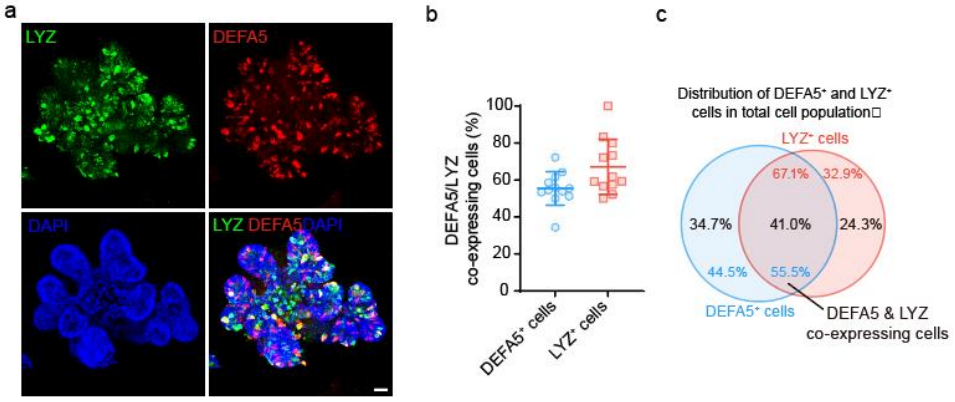
3. *LYZ* mRNA expression pattern showed widespread expression pattern across datasets:

To further substantiate our findings, we expanded our analysis to include *LYZ* expression patterns from other datasets alongside our own (TpC dataset). In studies such as Fujii et al., 2018 (ES, IF datasets) and He et al., 2022 (IL22 datasets), where Paneth cells are rare, or in the Crypt dataset comprising freshly isolated human intestinal crypts, *LYZ* mRNA is still observable in multiple cell types (**Rebuttal Fig. 7**). Notably, *LYZ* was not explicitly analyzed

or used as a marker for Paneth cells in these referenced studies.



Rebuttal Fig.7 *LYZ* expression in sc-RNAseq datasets



Rebuttal Fig.8 Co-localization of LYZ and DEFA5 proteins

We hypothesize that the widespread expression of *LYZ* mRNA might be influenced by Wnt

signaling, a common factor in all human intestinal organoid culture systems. Moreover, the particular enrichment of Lysozyme protein in Paneth cells, as opposed to its widespread mRNA expression, may be attributed to cell-specific post-translational regulation mechanisms.

7. Fig.3a, b: To analyze the function of those three small molecules, the authors tested the effect of stemness and the ability to differentiate organoids by excluding factors one by one. When each factor, pVc or CP, was removed from the complete TpC medium (Those condition mediums contained CHIR+TSA+CP (-pVc) or CHIR+TSA+pVc (-CP), respectively), the number of stem cells decreased, as shown here. However, this result confused me greatly because this represented data is the opposite compared with Extended Data Fig.3 and the previous report (ref. 22, Yin et al. Nat Methods). The authors and Yin et al. demonstrated that the number of Lgr5+stem cells was increased in the organoids cultured in the presence of CHIR (Wnt agonist) and histone deacetylase inhibitors (VPA, TSA). The authors need to explain this discrepancy.

Furthermore, I also could not follow why the organoid growth was inhibited in the culture condition -CP because the medium still contains Wnt agonist, EGF, RSPO, BMP inhibitor, A83-01, IGF-1, FGF-2 that is almost the same as IF medium. Does TSA or pVc alone affect organoid growth?

Response:

Thank you for this question. We understand the need for clarity regarding the function of the three small molecules (TSA, pVc, CP) and their effect on stemness and differentiation. Here we aim to provide a clear explanation of our results:

1. Experimental design and Basal vs. TpC conditions:

We used Basal and TpC conditions as starting points to evaluate the effects of TSA, pVc, and CP. The Basal condition, similar to the IF condition but with CHIR replacing Wnt3a conditioned medium, is not sufficient to maintain LGR5 stem cells in human intestinal organoids, even when TSA is added (CHIR+TSA), although it's sufficient to maintain mouse Lgr5 stem cells. This highlights the differences between mouse and human intestinal cells. In human intestinal organoids, CHIR is still essential as it significantly increases cell survival and proliferation but is not sufficient alone.

In contrast, the TpC condition, which includes TSA, pVc, and CP added to the Basal condition,

achieved the highest LGR5 maintenance, cell proliferation, and cellular diversity in the organoids.

2. We use two experimental approaches to evaluate the effect of the molecules:

Exclusion Experiments: Removing single or combinations of factors from the TpC condition.

Addition Experiments: Adding single or combinations of factors to the Basal condition.

We measured multiple metrics, including cell proliferation, the proportion of LGR5 cells, cellular diversity, colony-forming efficiency, and budding-structure formation.

3. Exclusion of molecules shows reduced performance than TpC but better than Basal:

Excluding CP from the TpC condition (TSA+pVc) resulted in a significant decrease compared to the complete TpC condition but also showed slight increases in LGR5 stem cells, cell proliferation, and colony formation compared to the Basal condition (**Fig. 3a-e**).

To summarize, we have reorganized the description in the revised manuscript:

"When TSA, pVc, and CP were added individually or in combination to the basal condition, TSA and CP significantly increased the LGR5-mNeonGreen percentages, and CP showing the most pronounced effect on cell proliferation, colony formation from single cells, and LGR5-mNeonGreen expression (Fig. 3a-e). TSA and CP also boosted the expression of Paneth cell markers (*DEFA5*, *DEFA6*, *LYZ*) and stem cell markers (*LGR5*, *SMOC2*), with the TpC condition showing the highest levels (Fig. 3f)."

4. Clarifying discrepancies:

The differences in our results compared to Yin et al. (Nat Methods) can be attributed to species-specific responses. In human organoids, CHIR+TSA is effective, but not sufficient to maintain LGR5 stem cells, whereas in mouse organoids, this combination is sufficient.

We hope this addresses your concerns.

8. Fig. 5m: The authors mentioned that Goblet cell differentiation was partially observed from the lineage of early enterocyte cells (shown in Result p13, lines 24-26). But which data supports this conclusion? It might help prove their idea if they could introduce the pure population of early enterocyte status and force into Goblet cell differentiation by inhibiting Notch signaling.

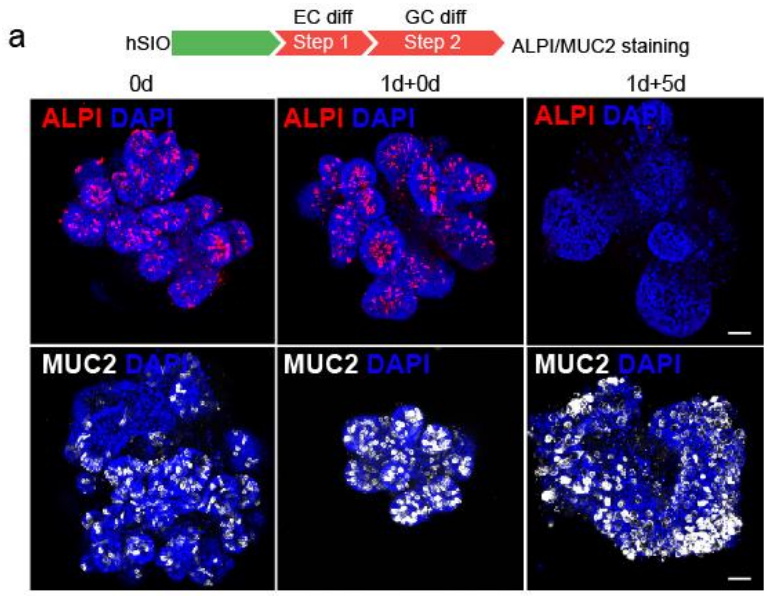
Response:

Thank you for this question. Our hypothesis that goblet cells can be generated from the lineage of early enterocytes is primarily based on observations from our scRNA-seq analysis.

In our scRNA-seq data, we identified two distinct secretory precursors. The first precursor expressed classical secretory markers, representing typical secretory precursors. The second precursor expressed *OLFM4* along with markers of both secretory and enterocyte lineages, suggesting these cells may represent transdifferentiated cells from enterocyte lineages. Additionally, the force atlas layout and PAGA trajectory analysis captured connections between early enterocyte cells (EC) and goblet cells (Supplementary Fig. 5b and Fig.2l-m), indicating a potential transition pathway. This may reflect cell fate transitions under organoid culture conditions, which maintain cells in a progenitor and more plastic state, rather than *in vivo*.

Following your advice, we attempted to verify this observation experimentally. Sorting a pure population of early enterocytes in human intestinal organoids using a specific marker is technically challenging, and maintaining the survival and differentiation of single cells post-sorting is difficult. Therefore, we performed a two-step differentiation process to induce the transition (**Rebuttal Fig. 9; new Supplementary Fig. 19**). We first induced EC differentiation for one day to enrich early EC, followed by Goblet cell differentiation for the next five days. As a result, we observed high-efficiency differentiation of goblet cells ($MUC2^+$), with reduced EC differentiation ($ALPI^+$). This experiment partially confirmed the presence of a connection between early EC and Goblet cells.

> *"Trans-differentiation from early EC cells to goblet cells was implied from scRNA-seq data (Fig. 2l-m and Supplementary Fig. 5b). Similarly, when we transferred partially differentiated enterocytes into goblet cell differentiation conditions, we observed a decrease in enterocyte differentiation but maintained goblet cell differentiation (Supplementary Fig. 19). Although this experimental data is not sufficiently conclusive to fully support this speculation, it further suggests the plasticity of cells in the organoids."*



Rebuttal Fig.9 The trans-differentiation from early enterocyte to goblet cell

Minor Comments:

1. Fig.1c: The original ES medium (Sato et al. Gastroenterology) does not contain PGE2.

Response:

Thank you for correction. We have modified this section, removed PGE2 and added omissive component Gastrin-I.

2. p12, line 15-16: As there are several reports previously that treatment DAPT in human intestinal organoids led to cell destiny towards secretory cells, please cite those papers.

Response:

We are grateful for this suggestion. We have cited related papers in the viewed manuscript.

"33. VanDussen, K.L., *et al.* Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. *Gut* **64**, 911-920 (2015).

34. Wang, Y., *et al.* Formation of Human Colonic Crypt Array by Application of Chemical Gradients Across a Shaped Epithelial Monolayer. *Cell Mol Gastroenterol Hepatol* **5**, 113-130 (2018).

35. Jung, P., *et al.* Isolation and in vitro expansion of human colonic stem cells. *Nat Med* **17**, 1225-1227 (2011)."

3. p13, line 4-6: Same as above, please cite the previous report that the Hans Clevers group have already demonstrated that inhibition of the cell cycle using Gefenitib induces enteroendocrine cell differentiation (Basak et al. Cell Stem Cell 2017).

Response:

Thank you for the suggestion. We have cited the paper in our viewed manuscript.

4. Methods (p16, line 16): There is no information about the approval number of this ethics.

Response:

Thank you for pointing out our omission. We have added the approval information in methods part in the final manuscript.

5. Methods (p17, line 22): Please put a space between the number and the unit in the part of EGF and IGF.

Response:

Thank you for point out this error. We have made revisions in the revised manuscript.

6. Sup Table 2: The information of that sequence might be unclear. They should put more information appropriately. Which part is for the homology arm for the LGR5 locus?

Response:

Thank you for your valuable feedback. We have updated the LGR5-reporter system sequence information in the new supplementary table 1.

Reviewer #2 (Remarks to the Author):

In the presented manuscript, Yang et al. provide a medium composition for the culture of adult stem cell-derived small intestinal organoids. Compared to other published media, the study presents a medium with higher secretory cell diversity and homeostatic stemness through the combination of previously described compounds and addition of three small molecules. Based on this medium, the authors explore how small intestinal cell types can be enriched through combinatorial inhibition/activation of certain pathways. While the manuscript clearly presents a push towards the secretory lineage, the downsides of this approach are not clearly enough addressed or at least in depth discussed in the current version. This includes the very low abundance of absorptive cell types, missing crypt-villus gradients of proliferation and cell-types (e.g. higher proliferation in the crypt), as well as phenotypic variability within cultures. While the manuscript presents intriguing findings and usable enrichment strategies of various cell types, it requires further mechanistic insights, addressing potential sources of noise, and especially proper comparisons to in vivo data to fully elucidate the cellular and molecular processes at play. Addressing these major and minor points will significantly enhance the scientific rigor and clarity of the study.

We thank the reviewer for reviewing our manuscript and providing valuable comments. We have taken these comments seriously and revised our manuscript to enhance the rigor of our work. The newly added contents are shown in rebuttal figure and highlighted in the final manuscript, and we hope our response sufficiently addresses your concerns. Thank you for your time and consideration.

Major Points:

1. Lack of Mechanistic Insight: The manuscript lacks mechanistic insight into the observed phenomena. Specifically, the manuscript mentions the use of HDAC inhibitors and their effects on chromatin rearrangements. It is important to investigate in general how these inhibitors affect the chromatin (over time). In more detail, one could try to resolve whether these inhibitors specifically target certain chromatin complexes (e.g., NuRD, SWI/SNF, PRC1/2) and how these effects relate to the de-differentiation and differentiation of intestinal stem cells. Overall, the potential role of HDAC inhibitors

in maintaining cells in a plastic/regenerative state should be explored.

Response:

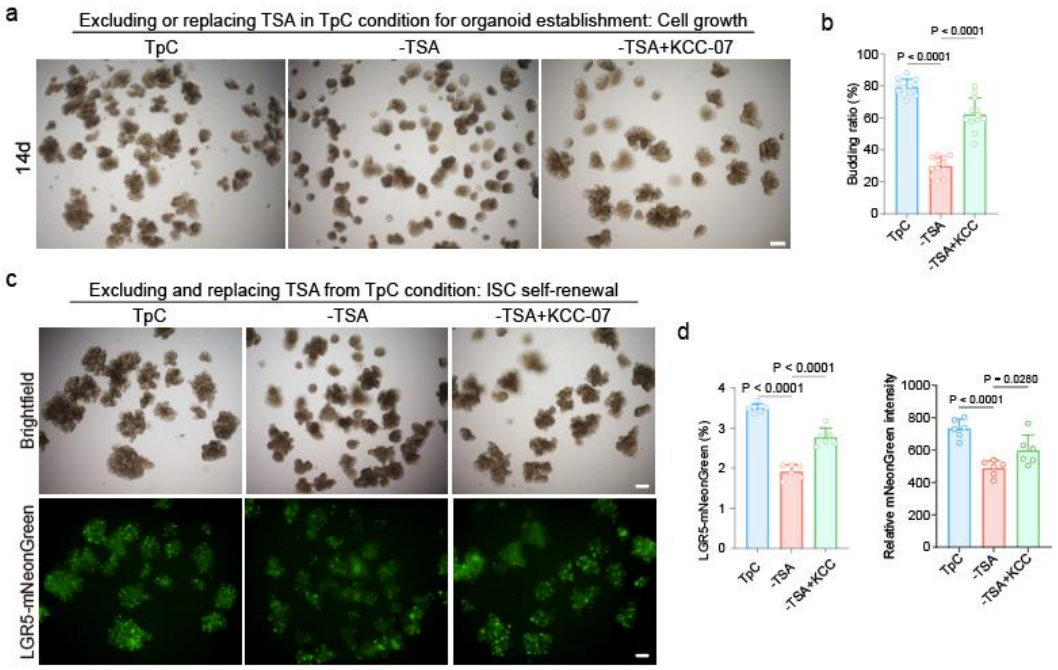
Thank you for this constructive suggestion. We have now provided a detailed analysis of the mechanism of HDAC inhibitors. Our findings are as follows:

1. TSA targets HDAC1/2 to promote stemness and cell diversity:

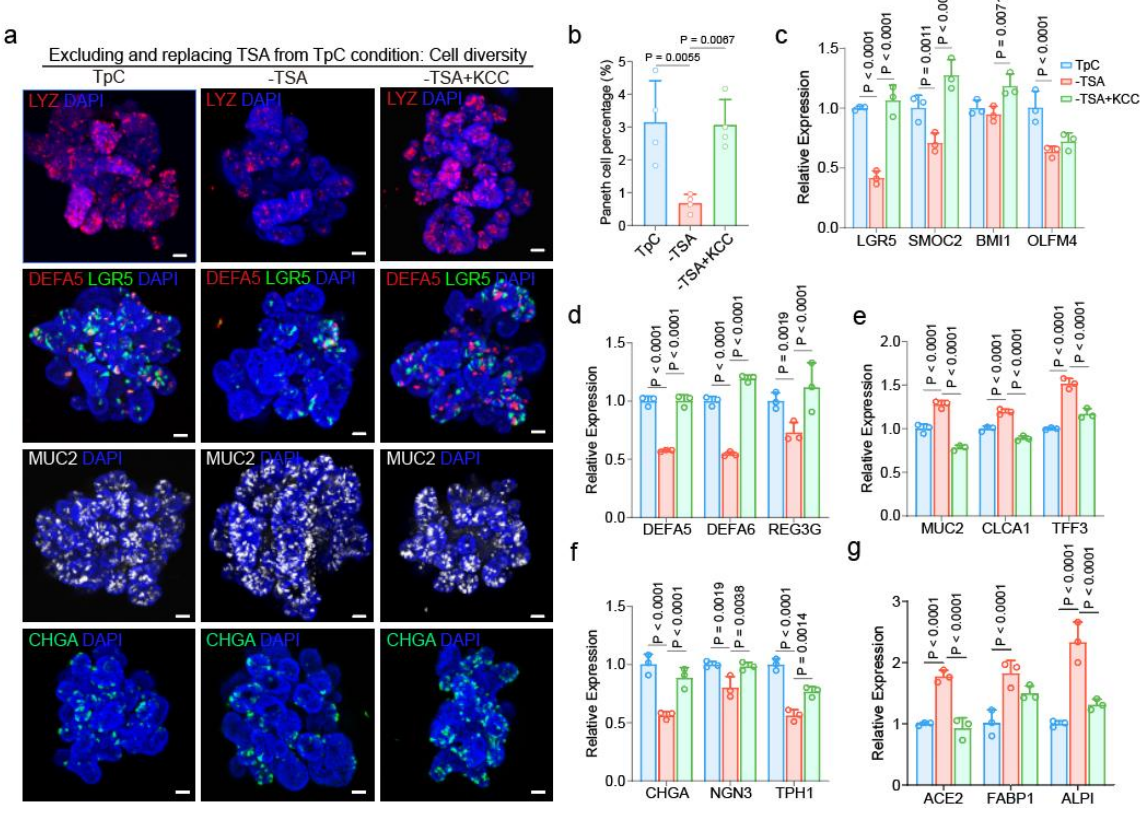
TSA is a pan-HDAC inhibitor. We first aim to identify the specific targets of TSA. We tested multiple HDAC inhibitors and observed their effects on LGR5-mNeonGreen expression and cellular diversity. The result showed that some inhibitors exhibited similar effects with different extent, with the HDAC2 inhibitor CAY10683 closely mimicking TSA's effects. This led to the conclusion that TSA likely targets HDAC1/2 to promote stemness and cell diversity (**Supplementary Fig. 11a-e in the original manuscript**).

2. Chemical inhibition of the NuRD complex subunit MBD2 mimics TSA effects:

To investigate the specific chromatin complexes affected by TSA, we tested inhibitors that modulate other subunits of complexes that contains HDAC1/2 subunit (e.g. NuRD). We found that KCC-07, an MBD2 inhibitor, rescued the phenotype of TSA removal, including budding ratio and LGR5-mNeonGreen expression (**Rebuttal Fig.10a-d; new Fig. 3p and new Supplementary Fig. 12a-d**). Furthermore, KCC-07 restored the abundance of secretory cells, especially Paneth cells compared to the -TSA condition (**Rebuttal Fig. 11a-b; new Supplementary Fig. 12e-f**), The expression of cell type-specific markers further confirmed the similar effects of KCC-07 and TSA (**Rebuttal Fig. 11c-g; new Supplementary Fig. 12g-k**). This shows that the NuRD complex plays a significant role in the effects observed with TSA treatment.



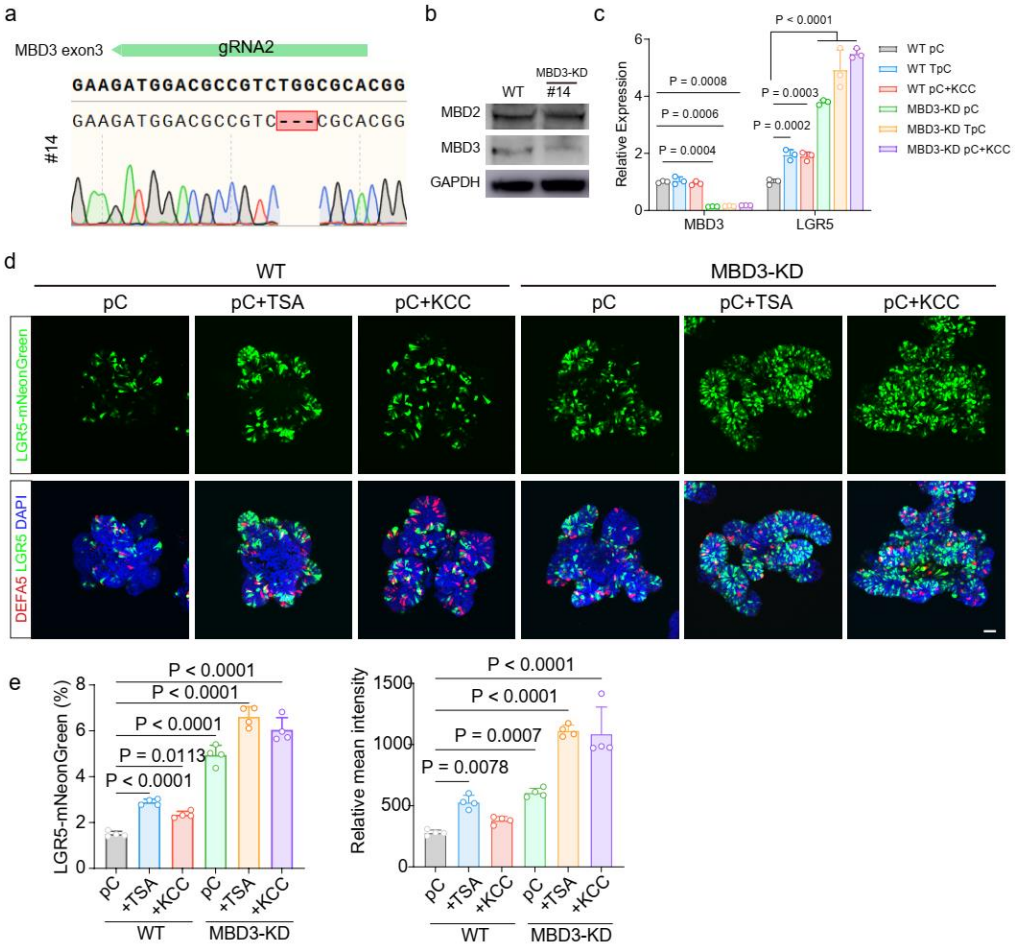
Rebuttal Fig. 10 KCC-07 rescued the budding ratio and LGR5-mNeonGreen proportion



Rebuttal Fig. 11 KCC-07 rescued Paneth cell abundance and cell type-specific gene expression

3. Genetic disruption of MBD3 shows similar effect as TSA:

The NuRD complex contain MBD2/3 subunits. Previously study has implied an inhibition role of MBD3-NuRD repressor complex in Lgr5 expression and progenitor proliferation in mouse intestine[1]. In the absence of specific MBD3 inhibitor, we used CRISPR/Cas9 to knock down MBD3 expression in human intestinal cells (**Rebuttal Fig. 12a-c; new Supplementary Fig. 13a-c)**. Knockdown of MBD3 significantly increased LGR5 expression and the proportion of LGR5-mNeonGreen stem cells, even under TSA or KCC-07 conditions. Simultaneous inhibition of both MBD2 and MBD3 (MBD3-KD + KCC-07) further enriched LGR5-mNeonGreen stem cells (**Rebuttal Fig. 12c-e; new Supplementary Fig. 13c-e and new Fig. 3r)**.



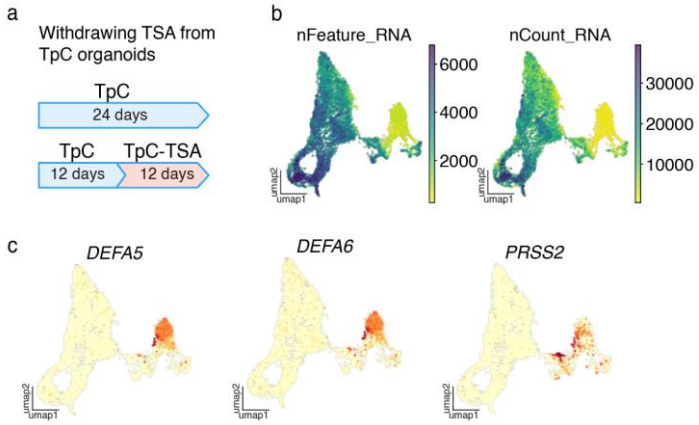
Rebuttal Fig. 12 MBD3 knockdown significantly increase the proportion of LGR5 stem cells

Reference:

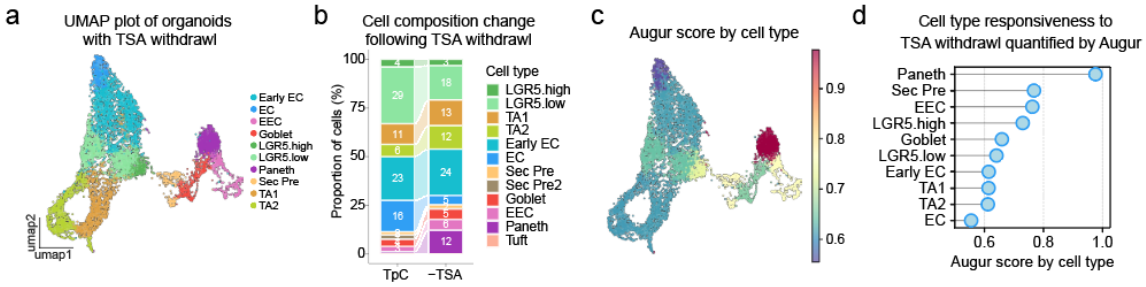
1. Aguilera, C., *et al*. c-Jun N-terminal phosphorylation antagonises recruitment of the Mbd3/NuRD repressor complex. *Nature* 469, 231-235 (2011).

4. TSA prevents premature differentiation of Paneth cells:

To further understand the effect of TSA on the organoids. We performed scRNA-seq on organoids following TSA withdrawal from already established TpC organoids (**Rebuttal Fig. 13; new Supplementary Fig. 14**). The results indicated that TSA withdrawal led to changes in cell differentiation, specifically showing an increase in the differentiation and subsequent death of Paneth cells (**Rebuttal Fig. 13, 14; new Supplementary Fig. 14 and new Supplementary Fig. 15a-d**). This suggests that TSA helps maintain a regenerative state by preventing premature differentiation of these cells.



Rebuttal Fig. 13 Effects of TSA withdrawal on TpC organoids



Rebuttal Fig. 14 Cellular changes in organoids following TSA withdrawal

In summary, these experiments confirmed that TSA, as a HDAC1/2 inhibitor in human

intestinal organoids, led to an MBD2/MBD3-NuRD complex-mediated LGR5 stem cell enrichment, which consequently enhance Paneth cell abundance.

Note: The phenotype observed upon TSA withdrawal from TpC organoids, specifically the increased differentiation of Paneth cells, is consistent with the phenotype of organoids initiated in media excluding TSA (pC condition), where a reduced abundance of Paneth cells is observed. In media without TSA, there are fewer LGR5 cells capable of generating Paneth cells, leading to reduced cell diversity. In contrast, TSA withdrawal causes the existing LGR5 cells in the TpC organoids to begin differentiating into more Paneth cells, thereby increasing cell diversity.

2. There seems to be a high discrepancy between cell type abundances in the scRNAseq (Figure 2) and imaging (Figure 1). For example, LYZ1+ cells are shown in very high abundance throughout the whole organoid in Figure 1, while only 1% of all cells are counted as Paneth cells in Figure 2. Could the authors comment on this discrepancy?

Response:

Thank you for raising this concern. We understand the discrepancy in cell type abundance between the immunostaining data (Figure 1) and scRNA-seq data (Figure 2) and would like to clarify that these differences do not indicate a contradiction but rather reflect methodological variations and inherent challenges in both techniques. This discrepancy can be attributed to several factors:

1. Preprocessing of single-cell samples and sampling bias:

The preparation of single-cell samples involves steps such as single cell dissociation, microfluidic partitioning, and library preparation, which can significantly impact cellular composition.

Mature cell types like Paneth cells are more fragile and prone to damage during these processes, leading to their loss during subsequent QC and filtering steps.

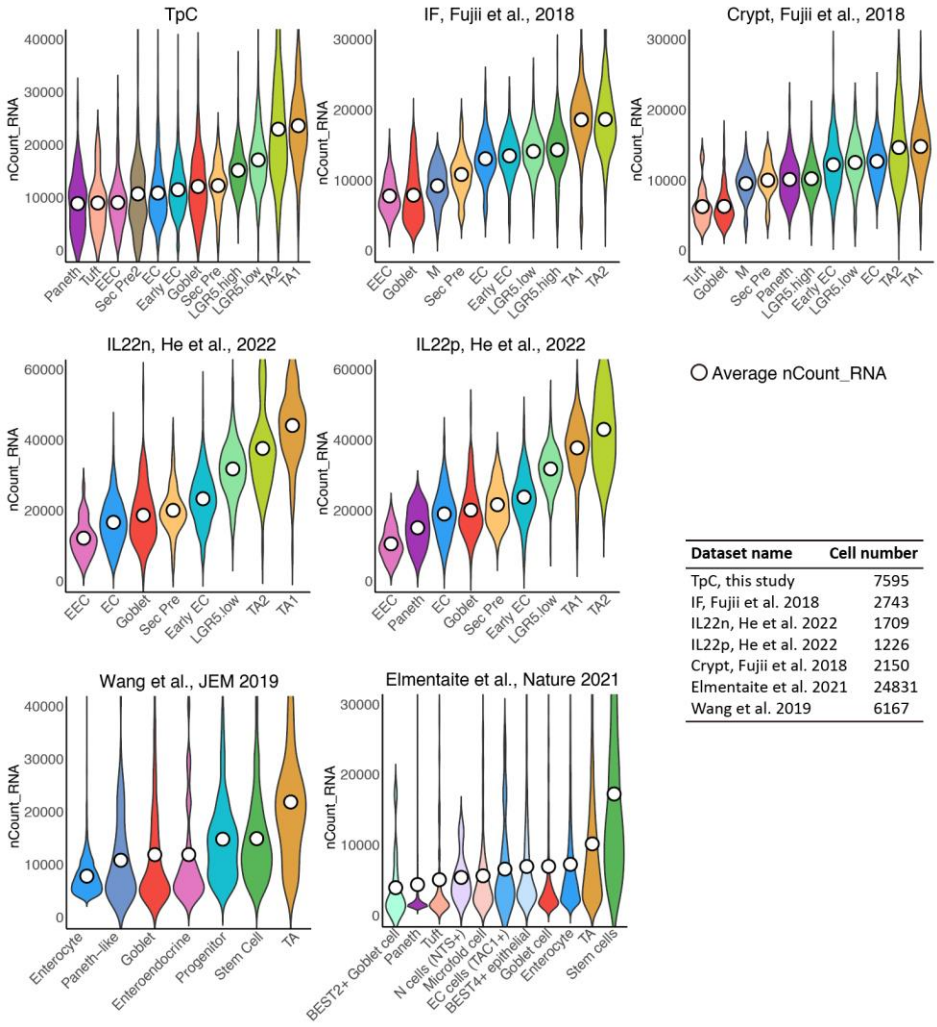
Cell dissociation for scRNA-seq can also introduce biases favoring cell types that dissociate more easily. In our organoids, stem cells and progenitor cells dissociate more readily than

mature, differentiated cells like Paneth cells, leading to their underrepresentation in scRNA-seq data.

Denisenko et al.[1] have systematically assessed protocols affecting cell composition during scRNA-seq, highlighting these biases. In addition, “both (10X and Smart-seq2) techniques have cell sampling bias that could affect the cell composition of scRNA-seq datasets. For example, human neutrophils dropout frequently occurs in 10X Chromium system, and fragile cells, such as macrophages and some types of stromal cells, tend to be lost during cell sorting because of high-pressure” [2].

2. Sequencing Depth:

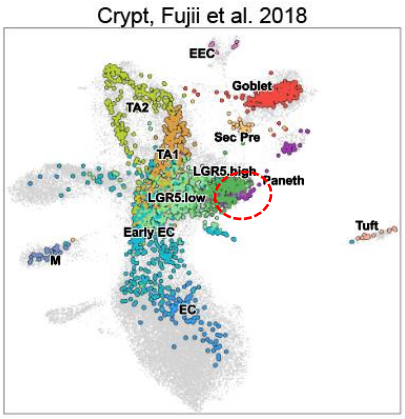
The depth of sequencing in scRNA-seq affects the detection of low-abundance cell types. Low-mRNA content cell types, such as Paneth cells, may be underestimated if sequencing depth is insufficient, which is often the case with 10x Genomics platforms. Our data show that mature cell types, specifically secretory cells, generally contain less RNA content, leading to their underrepresentation (**Rebuttal Fig. 15**).



Dataset name	Cell number
TpC, this study	7595
IF, Fujii et al. 2018	2743
IL22n, He et al. 2022	1709
IL22p, He et al. 2022	1226
Crypt, Fujii et al. 2018	2150
Elmentaite et al. 2021	24831
Wang et al. 2019	6167

Rebuttal Fig. 15 RNA counts of different cell types

Note: in the Crypt sample, Paneth cell cluster together with LGR5 cells, which likely also includes partially differentiation LGR5 cells (See **Supplementary Fig.6b**).

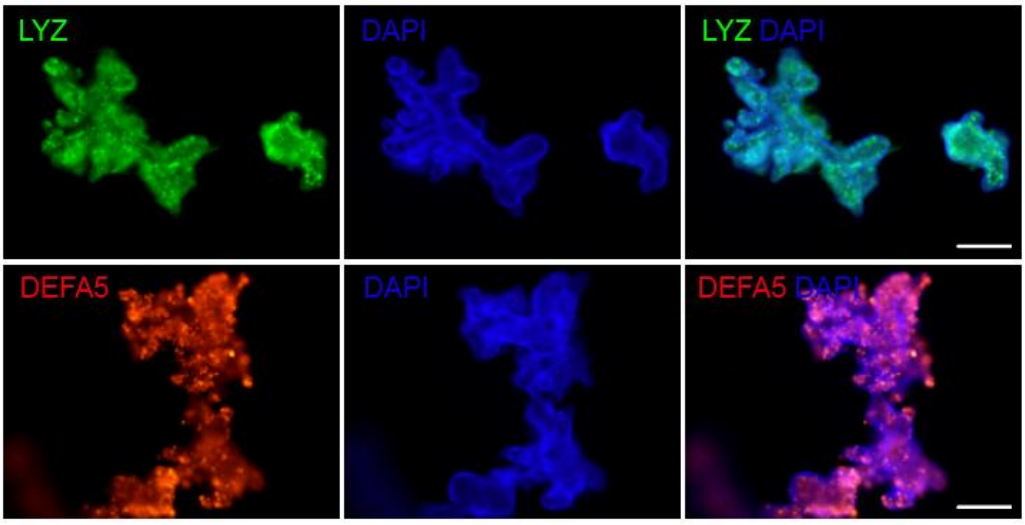


3. Sample Variation:

Organoids are highly heterogeneous and dynamic populations of cells. Cell composition can change in response to culture conditions and time, making it challenging to achieve stable cell composition across different batches or different culturing time. This variability contributes to discrepancies between different experimental approaches.

4. Supporting Evidence:

We have provided staining of Paneth cell markers for the exact same sample during our QC step before performing scRNA-seq. This validates the presence and abundance of Paneth cells in our organoids, corroborating the immunostaining data (**Rebuttal Fig. 16**).



Rebuttal Fig. 16 Quality control by staining before performing scRNA-seq

We appreciate your comment and hope this detailed explanation addresses your concern.

References:

1. Denisenko, E., *et al.* Systematic assessment of tissue dissociation and storage biases in single-cell and single-nucleus RNA-seq workflows. *Genome Biol* **21**, 130 (2020).
2. Shichino, S., *et al.* TAS-Seq is a robust and sensitive amplification method for bead-based scRNA-seq. *Commun Biol* **5**(2022).

3. The UMAP representation of OLFM4 and ASCL2 expression appears inconsistent with their

known roles as master transcriptional regulators. Especially OLFM4 shows widespread patterns. The authors should explain whether these genes are still functioning as regulators and provide details on when (day of organoid development) the single-cell RNA sequencing experiment was conducted.

Response:

We appreciate your insightful comment regarding the expression patterns of OLFM4 and ASCL2 in our study. Below, we provide additional analysis for the role of OLFM4 and ASCL2.

1. Expression Patterns of OLFM4 and ASCL2:

We performed scRNA-seq on TpC organoid cells at 28 days of culture from single cells. Our analysis revealed that OLFM4 is not a precise marker for LGR5 stem cells in human intestinal cells. OLFM4 exhibited widespread expression across LGR5 stem cells, transit-amplifying (TA) cells, and some early enterocytes (Early EC), with the highest expression in LGR5-low stem cells. Conversely, ASCL2 showed a more restricted expression pattern, closely aligning with LGR5 but also present in some secretory cell types.

2. Supporting Literature:

Griffin et al. (2022) noted that OLFM4 is not a precise marker for human intestinal stem cells, with widespread expression across almost all crypt cells in the human small intestine.

Jang et al. (2015, 2016) reported OLFM4 expression in most intestinal crypt cells and TA cells in the upper half of crypts, while ASCL2 colocalized closely with LGR5 at the crypt base.

Ziskin et al. (2013) showed that OLFM4 does not colocalize with LGR5 in normal human colon tissue, exhibiting a more diffuse distribution.

Gersemann et al. (2012) found OLFM4 expression extending to mid-crypt in normal human colon mucosa, above the stem cell niche.

3. Additional Data Analysis:

Our additional analysis of OLFM4 and ASCL2 expressions in single-cell datasets from Fujii et al. (2018) and He et al. (2022) further corroborates these findings. OLFM4 showed

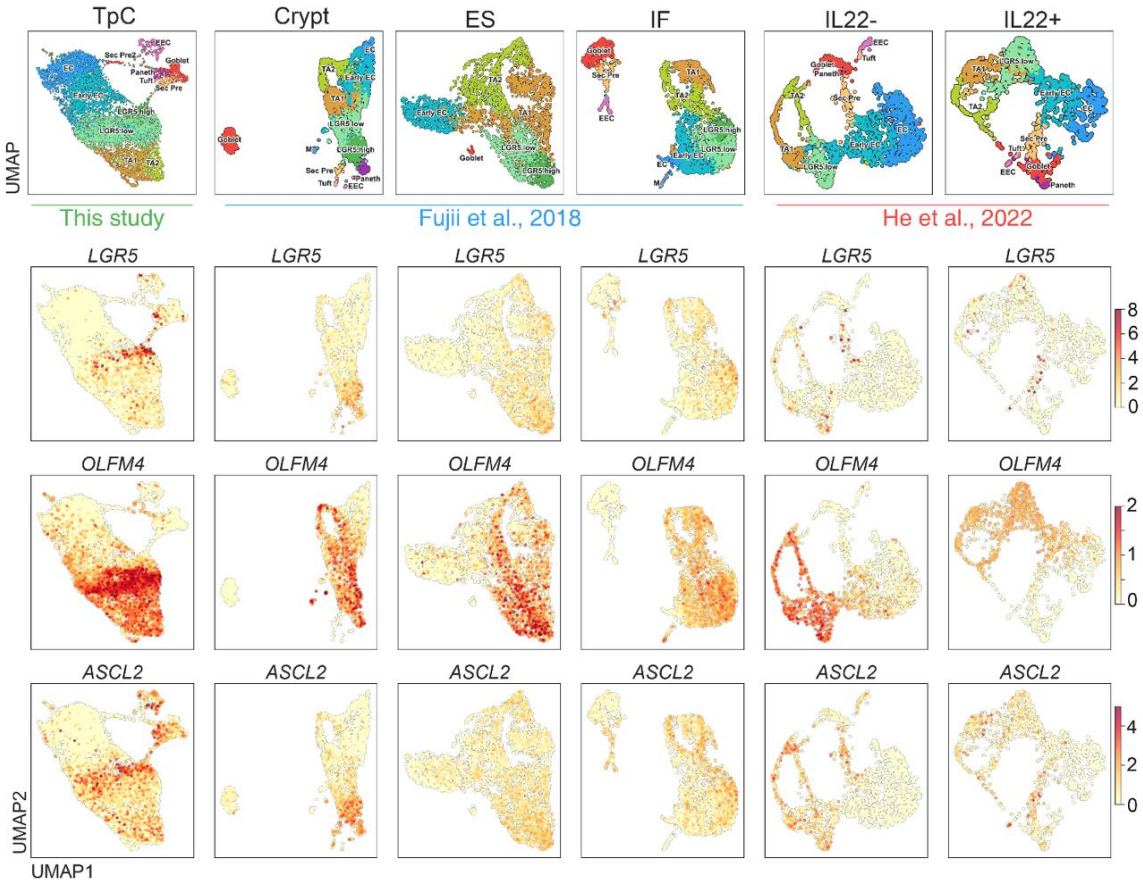
widespread expression across LGR5 stem cells, TA cells, and Early EC cells, while ASCL2 expression closely aligned with LGR5 but was broader (**Rebuttal Fig. 17**).

4. Conclusion:

Our findings, supported by literature and additional data analysis, suggest that ASCL2 acts as a more accurate indicator and potential master regulator of LGR5 stem cell states, while OLFM4's widespread expression limits its specificity as a master regulator in human intestinal cells.

Detailed Experimental Protocol:

To address your query about the timing of our single-cell RNA sequencing experiment, we have included a detailed description of the scRNA-seq protocol and its timing in the revised manuscript.



Rebuttal Fig. 17 OLFM4 and ASCL2 expressions in single-cell datasets

References:

Liu, W. & Rodgers, G. P. Olfactomedin 4 Is Not a Precise Marker for Human Intestinal Stem Cells, but Is Involved in Intestinal Carcinogenesis. *Gastroenterology* **162**, 1001–1004 (2022).

Gersemann, M., *et al*. Olfactomedin-4 is a glycoprotein secreted into mucus in active IBD. *Journal of Crohn's and Colitis* **6**, 425–434 (2012).

Jang, B. G., *et al*. Distribution of intestinal stem cell markers in colorectal precancerous lesions. *Histopathology* **68**, 567–577 (2016).

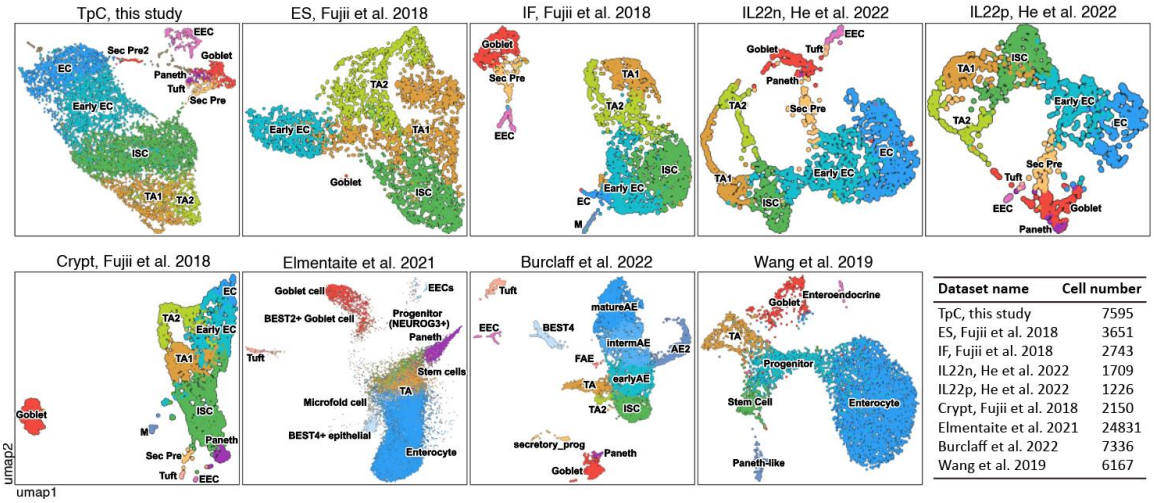
Jang, B. G., Lee, B. L. & Kim, W. H. Olfactomedin-related proteins 4 (OLFM4) expression is involved in early gastric carcinogenesis and of prognostic significance in advanced gastric cancer. *Virchows Arch* **467**, 285–294 (2015).

Ziskin, J. L., *et al*. In situ validation of an intestinal stem cell signature in colorectal cancer. *Gut* **62**, 1012–1023 (2013).

4. To enhance the significance of their medium composition, the authors should compare their results to published in vivo datasets to demonstrate the relevance and applicability of their findings.

Response:

We appreciate your suggestion to compare our results with published in vivo datasets to enhance the significance of our findings. We have performed additional comparison with 3 publicly available scRNA-seq datasets for in vivo human intestinal epithelial cells: GSE125970 (Wang et al., *J Exp Med*, 2020), E-MTAB-9543 (Elmentaite et al., *Nature*, 2021), and GSE185224 (Burclaff et al., *Cell Mol Gastroenterol Hepatol*, 2022) (**Rebuttal Fig. 18; new Supplementary Fig. 6a**).



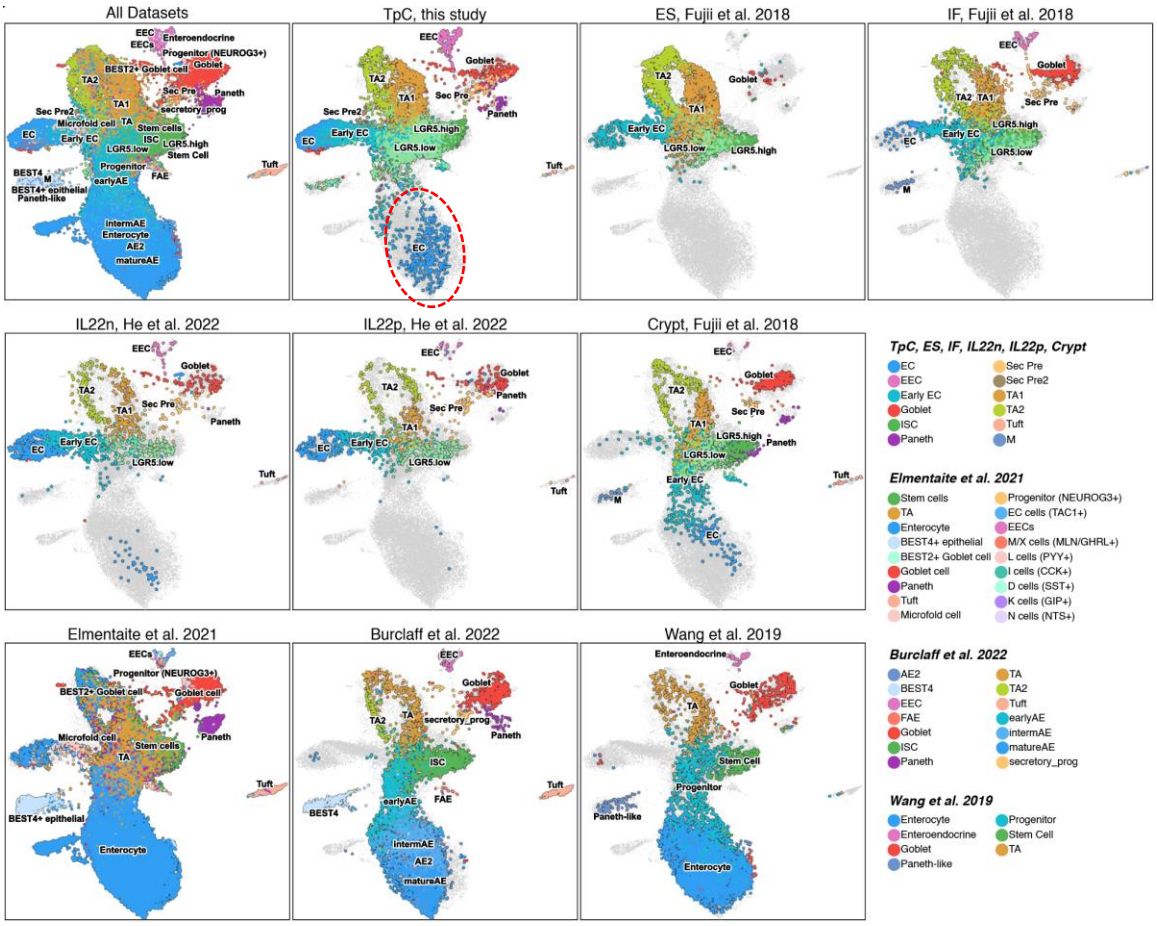
Dataset name	Cell number
TpC, this study	7595
ES, Fujii et al. 2018	3651
IF, Fujii et al. 2018	2743
IL22n, He et al. 2022	1709
IL22p, He et al. 2022	1226
Crypt, Fujii et al. 2018	2150
Elmentaite et al. 2021	24831
Burclaff et al. 2022	7336
Wang et al. 2019	6167

Rebuttal Fig. 18 UMAP plot of single-cell RNA-seq datasets

Below, we summarize the key findings:

1. Harmony integration with *in vivo* data

We integrated our organoid datasets with previous organoid datasets and *in vivo* datasets using Harmony for data integration. This allowed us to create a unified dataset to compare cell cluster distributions while preserving the original cell type annotations for the *in vivo* datasets. Our findings indicate that the cell types identified in our organoid cultures are consistent with those in the *in vivo* datasets. Notably, EC lineage cells formed distinct clusters with the *in vivo* datasets across all organoid conditions, likely due to the culture conditions with uniformly high Wnt and low BMP levels. But only our TpC organoids contained a significant number of EC cells that clustered with *in vivo* ECs (**Rebuttal Fig. 19; new Supplementary Fig. 6b**).



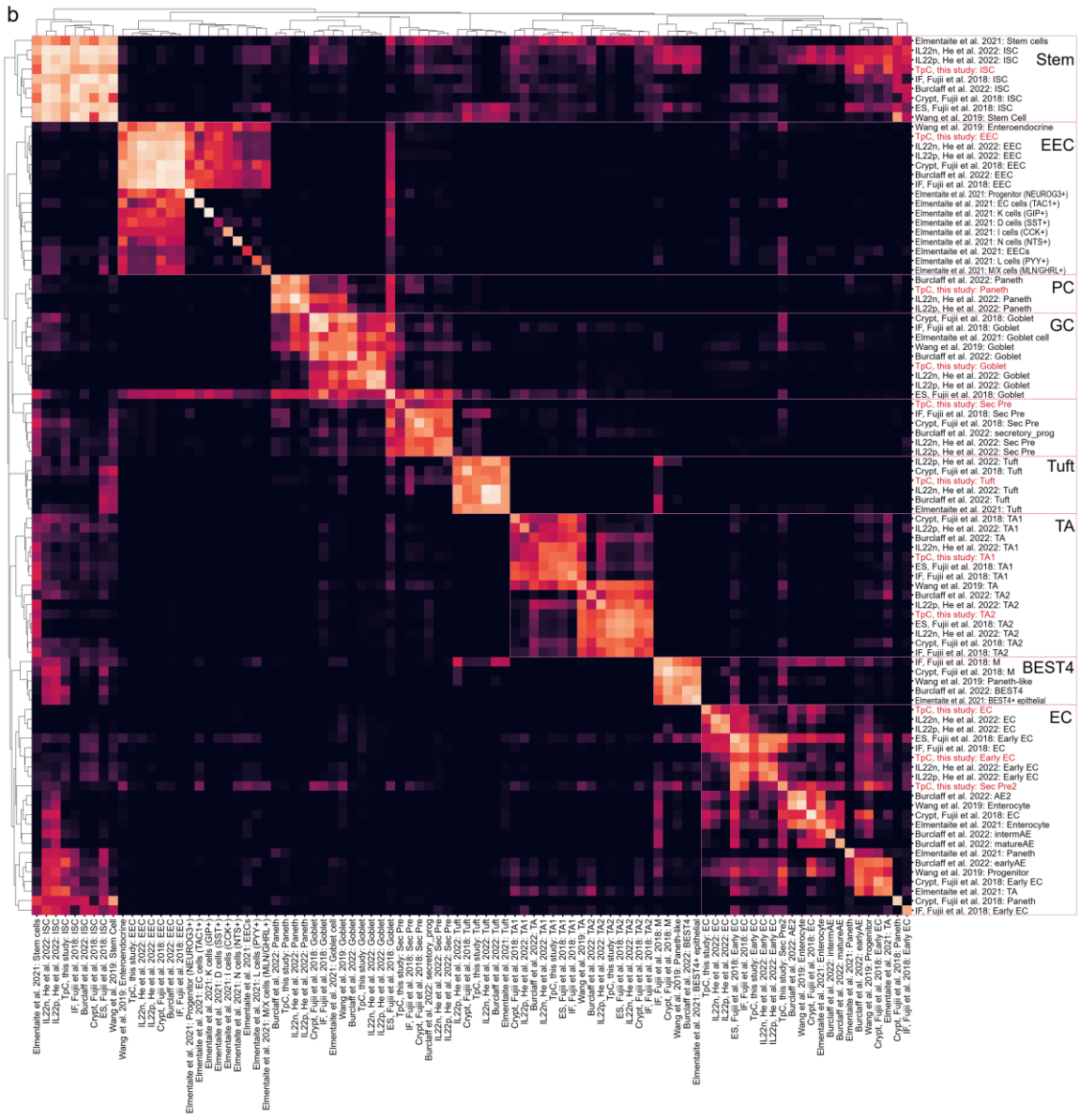
Rebuttal Fig. 19 Harmony integration of single-cell RNA-seq datasets from organoid and *in vivo* conditions

2. CellHint harmonization of cell annotations confirms consistency across studies

We utilized CellHint for cell type harmonization, generating tree plots and heatmaps to compare cell type assignments across different studies. This confirmed the consistency of the cell types identified in our organoid cultures with those in the *in vivo* datasets. Additionally, previously identified M cells in the IF/Crypt dataset co-clustered with BEST4 cells, consistent with the Harmony data. This further supporting the reliability of our cell type annotations **Rebuttal Fig. 20; new Supplementary Fig. 7-8**).

a

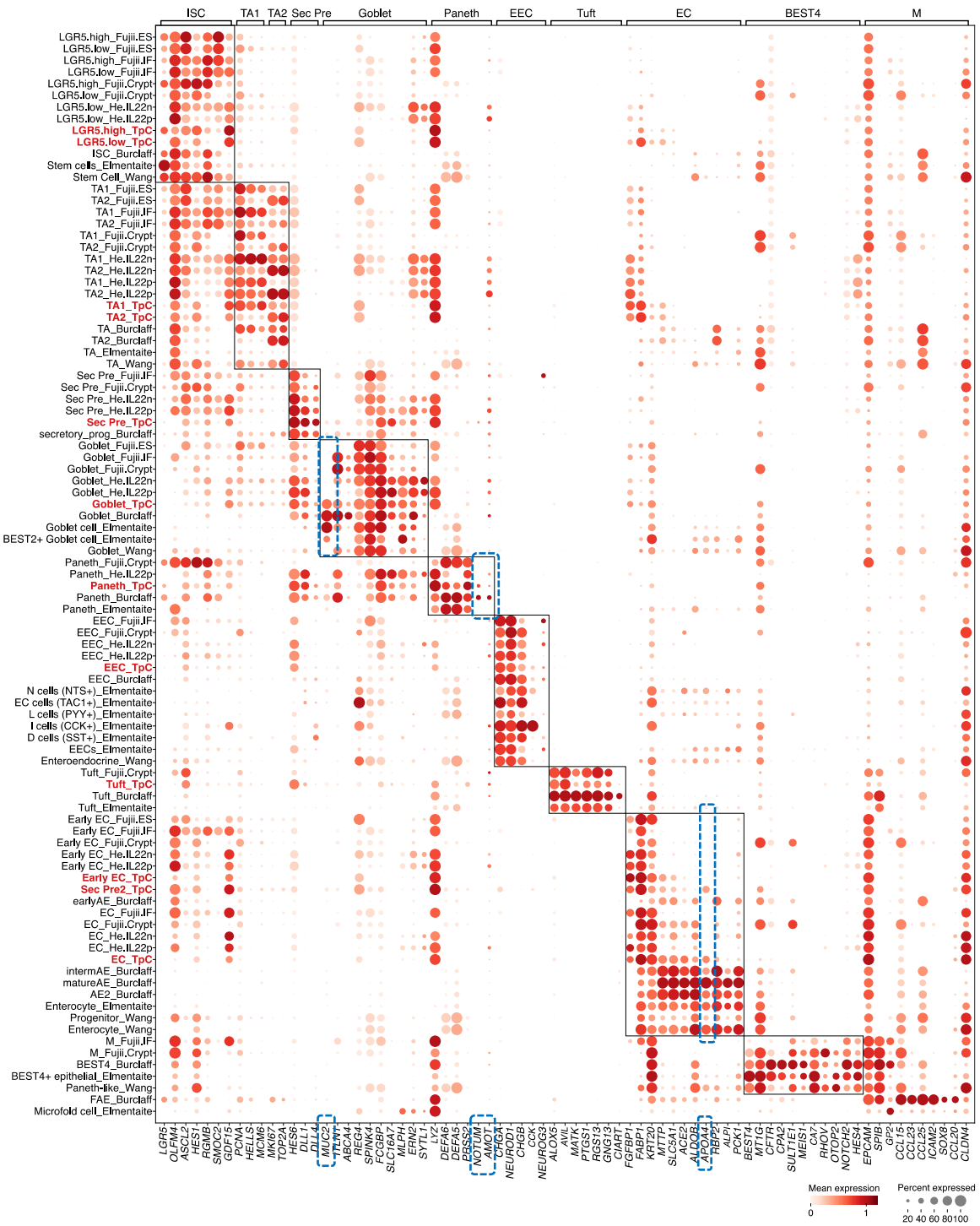

CellHint label harmonization tree
Crypt, Fujii et al. 2018
ES, Fujii et al. 2018
IF, Fujii et al. 2018
IL22n, He et al. 2022
IL22p, He et al. 2022
TpC, this study
Burclaff et al. 2022
Elmentaite et al. 2021
Wang et al. 2019
Early EC
EC
Sec Pre2
Goblet
Sec Pre
secretory_prog
EEC
Progenitor (NEUROG3+)
EC cells (TAC1+)
Enteroendocrine
N cells (NTS+)
FAE
Microfold cell
I cells (CCK+)
M/X cells (MLN/GHRL+)
Goblet cell
Paneth
BEST2+ Goblet cell
Tuft
earlyAE
TA
ISC
Stem cells
Stem Cell
AE2
K cells (GIP+)
matureAE
M
BEST4
BEST4+ epithelial
Paneth-like
Progenitor
intermAE
Enterocyte
TA2
TA1
D cells (SST+)
EECs
L cells (PYY+)



Rebuttal Fig. 20 CellHint-based harmonization of organoid and *in vivo* single-cell datasets

3. Marker gene expression supports similarity to *in vivo* cells

The marker gene expression in our organoids was similar to that in *in vivo* cell types, with TpC organoids showing the highest similarity. Examples include *MUC2* for goblet cells, *NOTUM* and *AMOT* for Paneth cells, and *APOA4* for ECs. This demonstrates the fidelity of our organoid system in recapitulating the cellular diversity of the intestinal epithelium (**Rebuttal Fig. 21; new Supplementary Fig. 9**).



Rebuttal Fig. 21 Marker expression in organoid and *in vivo* single-cell clusters

Conclusion

These findings highlight the robustness of our organoid system in mimicking *in vivo* cellular diversity, particularly over existing organoid conditions. We believe these comparisons

significantly enhance the relevance and applicability of our findings.

Reference:

Wang, Y., *et al*. Single-cell transcriptome analysis reveals differential nutrient absorption functions in human intestine. *J Exp Med* **217**(2020).

Elmentaite R., *et al*. Cells of the human intestinal tract mapped across space and time. *Nature* **597**, 250-255 (2021).

Burclaff J., *et al*. A Proximal-to-Distal Survey of Healthy Adult Human Small Intestine and Colon Epithelium by Single-Cell Transcriptomics. *Cell Mol Gastroenterol Hepatol* **13**, 1554-1589 (2022).

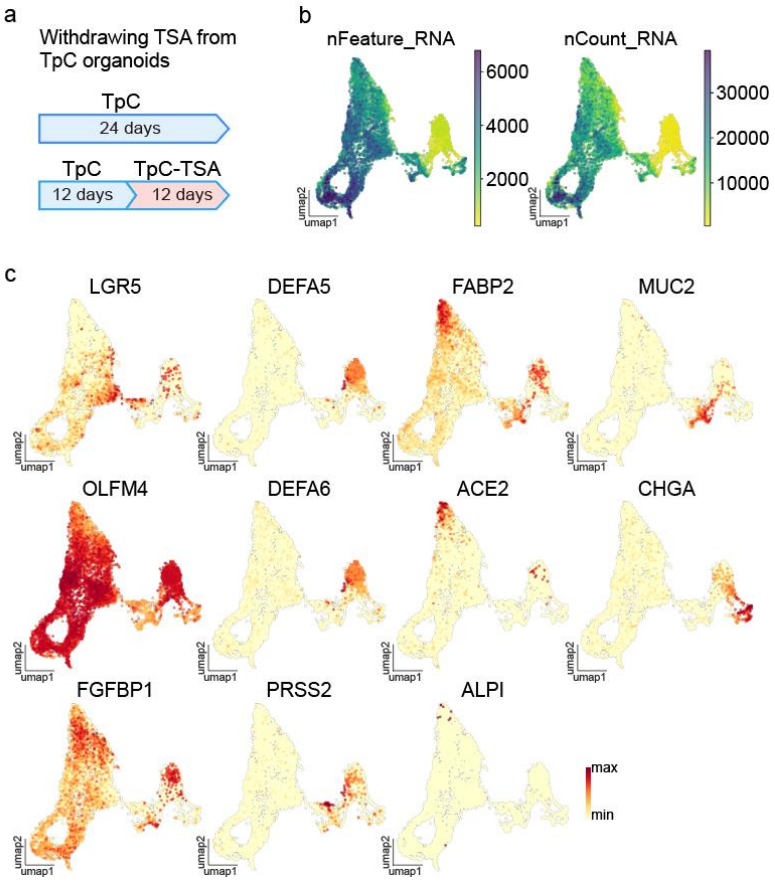
5. To clarify the impact of iBET inhibitors and TSA, perform additional sequencing experiments on LGR5-high, medium, low, and negative cells. This could help identify the specific cell types and processes affected by these treatments.

Response:

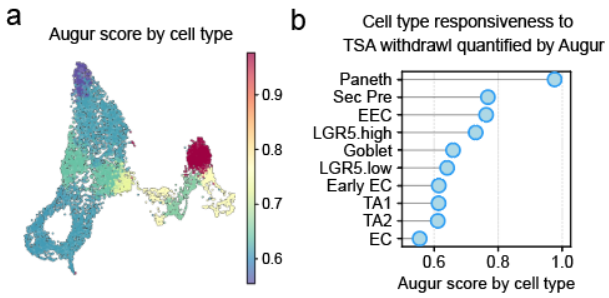
Thank you for your valuable comment. We conducted single-cell RNA-seq on iBET and TSA-treated organoids. This approach allowed us to comprehensively analyze the effects on all cell types, including those with varying LGR5 expression levels, within their native cellular context. Below, we summarize the key findings:

1. TSA withdrawal affects Paneth and LGR5-high cells, and prevent differentiation of LGR5 cells:

Single-cell RNA-seq of TSA withdrawal from TpC organoids revealed premature differentiation and death of Paneth cells, indicated by low UMI counts and feature numbers in cells expressing Paneth markers (*DEFA5*, *DEFA6*, *PRSS2*) (**Rebuttal Fig. 22; new Supplementary Fig. 14**). Using Augur, we found that Paneth cells, secretory precursors, and LGR5-high cells were most affected by TSA withdrawal (**Rebuttal Fig. 23; new Supplementary Fig. 15c-d**), consistent with TSA's role in maintaining LGR5 cells and preventing premature differentiation.



Rebuttal Fig. 22 Effects of TSA withdrawal on TpC organoids

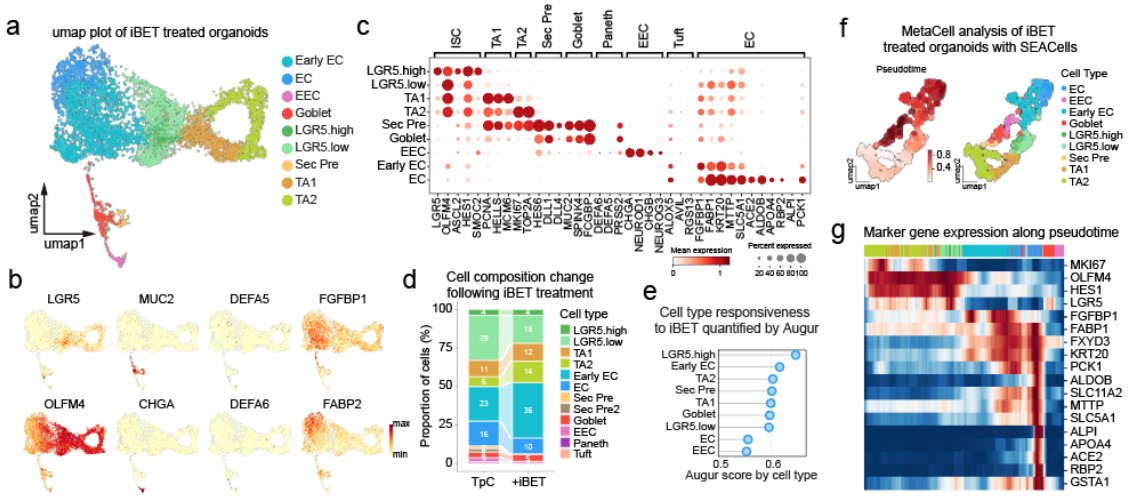


Rebuttal Fig. 23 Augur score by cell type

2. iBET treatment shift differentiation trajectory from secretory to EC lineages:

Single-cell RNA-seq of TpCI-cultured organoids identified most cell types found in TpC organoids, except Paneth cells. Secretory cells (EECs and precursors) were less abundant, while goblet cells remained similar (**Rebuttal Fig. 24a-c; new Fig 4g-i**). There was a notable increase in enterocyte lineage and TA cells (**Rebuttal Fig. 24d; new Fig 4j**). LGR5-high and

early enterocyte (EC) cells were most affected by iBET, shifting differentiation from secretory to enterocyte lineage (**Rebuttal Fig. 24e; new Fig 4k**). Trajectory analysis confirmed a pathway from TA cells to enterocytes mediated by LGR5 cells, indicating that iBET promotes enterocyte proliferation at the expense of secretory cell differentiation (**Rebuttal Fig. 24f-g; new Fig 4l-m**).



Rebuttal Fig. 24 scRNA-seq analysis of TpCI-cultured organoids

Conclusion:

These data clarify the impact of TSA and iBET on different cell types and the processes affected by these treatments. TSA withdrawal leads to premature differentiation and death of Paneth cells, significantly affecting LGR5-high cells. In contrast, iBET shifts differentiation towards enterocyte lineage, promoting their proliferation and reducing secretory cell differentiation. These results are highly consistent with our cellular phenotype data, further confirming the fidelity of our system.

6. As an effect of the extensive inhibitors used in the presented manuscript, it likely destroys any intrinsic crypt-villus gradient as e.g. ISC and paneth cells seem to be rather randomly distributed throughout the organoid (e.g. Figure 1i, m). This should be clearly noted in the manuscript as a disadvantage of this protocol. Currently, at page 6 lines 2-6 it reads as “high degree of homogeneity”, which sounds positive.

Response:

Thank you for this insightful comment. We have noted the shortcoming of the current protocol and have further clarified the description of “high degree of homogeneity.”

1. We acknowledge that future efforts are essential to achieve correct structure:

Regarding the randomly distributed Paneth cells, we agree this remains a challenge in the current organoid system. This distribution is a compromise to maintain the proliferative and differentiation capacity of stem cells in a homogeneous culture system like TpC. Generating multiple cell types with spatial organization requires localized signaling gradients, as seen *in vivo*. This challenge is inherent in adult stem cell-derived organoid systems where homogeneous culture media lack the spatial niche gradients found *in vivo*.

Our study primarily addresses the cellular diversity issue in existing human intestinal organoid systems. As discussed in our introduction:

> *“Generating diverse and rapidly proliferating cells necessitates stem cells with the capacity to generate multiple cell types and orchestrate localized signaling gradients for spatially regulated self-renewal and differentiation. Achieving this remains challenging in homogeneous organoid cultures lacking the spatial niche gradients found in vivo. In this study, we hypothesize that enhancing organoid stem cell stemness can amplify their differentiation potential, which would increase the cellular diversity in organoids without applying artificial spatiotemporal signaling gradients.”*

This was also elaborated in the last paragraph of our discussion section:

> *“Ultimately, creating an in vitro model that fully emulates in vivo physiology requires reproducing the capacity of stem cells to respond to complex spatiotemporal cues governing their self-renewal and differentiation, where a homogenous culture environment may limit the maturation of differentiated cells. Thus, we envision that artificially generating signaling gradients through bioengineering approaches could further improve the fidelity of organoid models.”*

We have now added a description acknowledging that our current TpC condition is still not sufficient to achieve the correct spatial structure for cell types as seen *in vivo* (the last paragraph of the Discussion section):

> *"Although our TpC system has increased cellular diversity in the organoids, it has not yet achieved the correct spatial structure and cell distribution as seen in vivo."*

2. The high degree of homogeneity is an advantage of our TpC culture system:

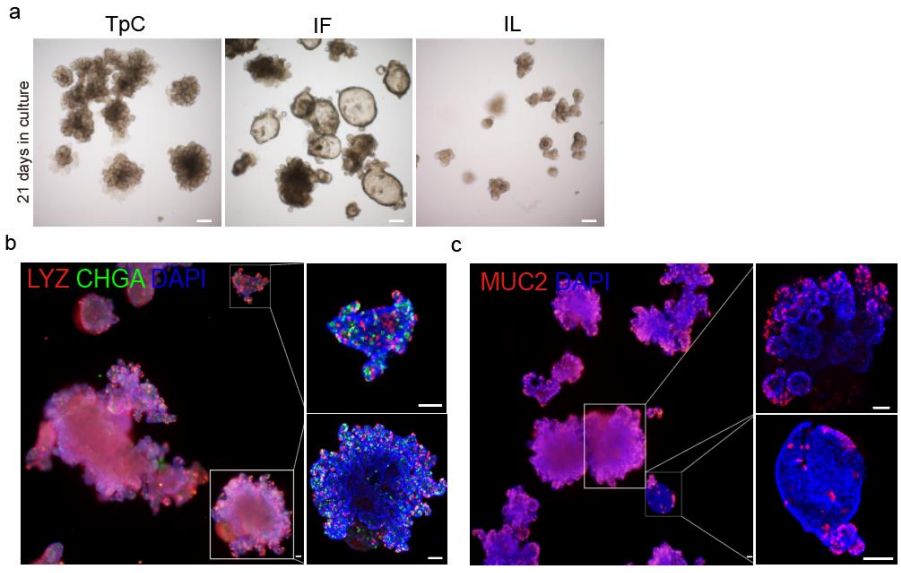
In our study, we observed a high degree of homogeneity in the composition (distribution of stem cells and differentiated cells) and structure (budding versus sphere) between organoids in the TpC system.

Typically, organoid cultures are characterized by high heterogeneity, posing significant challenges for reproducibility and consistency in experimental results. **Rebuttal Fig. 25a** (**new Supplementary Figure 2a**) demonstrates the variability observed in existing organoid cultures, highlighting their inherent heterogeneity. In contrast, our protocol results in more homogeneous cultures between different organoids, enhancing the reproducibility and reliability of our experimental outcomes. This homogeneity ensures that the organoids are more uniform in composition, making them more suitable for downstream applications such as high-throughput screening and comparative studies.

We have further clarified this by revise the text as:

> *"This indicates a high degree of homogeneity between TpC-generated organoids in terms of composition and structure, which is an advantage for downstream applications."*

Please also see our response addressing the comment of reviewer 1, major comment 3, where only a small number of round/cyst organoids possess heterogeneous distribution of secretory cells (**Rebuttal Fig. 25b-c; new Supplementary Fig. 3d-e**).



Rebuttal Fig. 25 Morphology and cell composition of organoids

Minor Points:

1. Inconsistent Organoid Sizes: The manuscript displays different organoid sizes in various figures. It would be beneficial to provide a representative scheme explaining why different sizes were used and how they relate to the experiments. Figure 1 shows notably larger organoids than Figure 4, and the authors should address the morphological differences observed.

Response:

Thank you for this comment. Our organoids are maintained by single-cell passaging, as shown in **Figure 1**. They can be cultured for 2-5 weeks before the next single-cell passaging and maintain relatively stable cell composition, as demonstrated in **Supplementary Fig. 2g-h**. We typically characterize our organoids when they form mature budding colonies at 3 to 5 weeks, unless otherwise indicated in the legends or figures (**e.g., Fig. 1l or Fig. 3a**).

To address your concern, we have updated the figures and legends to indicate the age of the organoids in culture. This should provide clarity on the relationship between the size of the organoids and the timing of their characterization in the experiments.

2. Phenotypic Heterogeneity: There seems to be large heterogeneity during culture, as captured for example in brightfield images of Figure 1d. Can the authors argue on the source of this heterogeneity and how their phenotypes are different from each other in terms of marker expression?

Response:

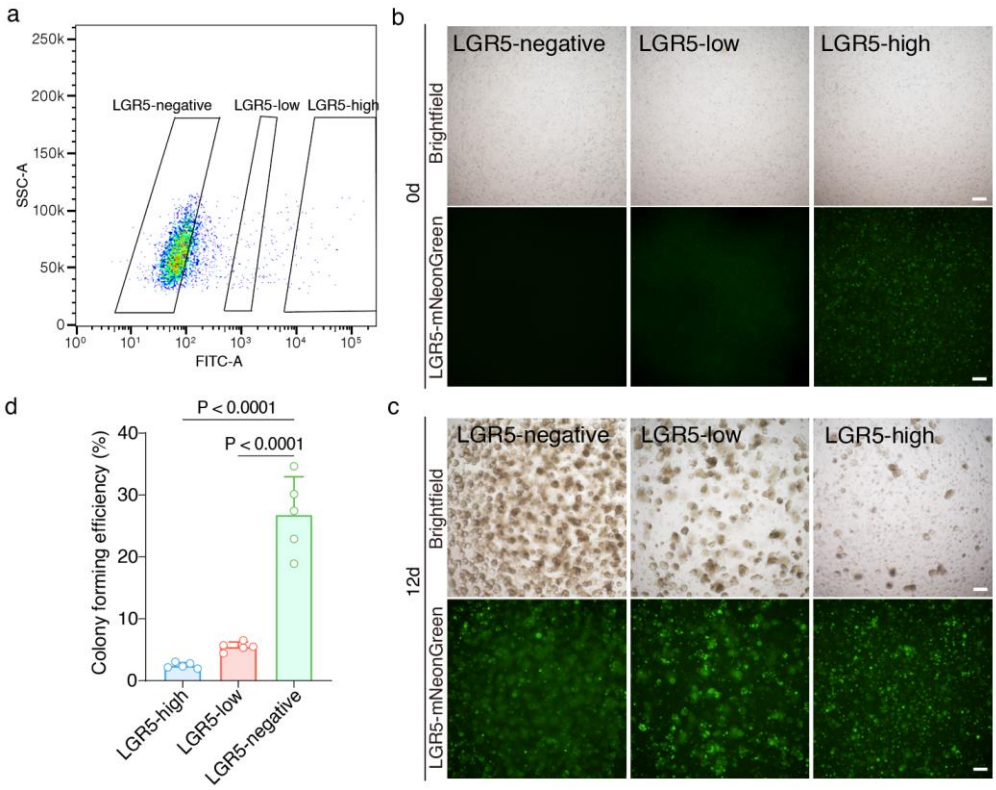
We appreciate your comment. In our TpC condition, we observed heterogeneity in shape/structure, size, and cell composition. Using immunostaining and single-cell organoid initiation experiments, we found that this heterogeneity primarily originates from the different types of cells that initiate the organoids starting from single cells.

Source of Heterogeneity:

Organoids can be initiated by multiple cell types, including LGR5-negative cells (such as TA cells and early EC cells) and LGR5-positive cells (**Rebuttal Fig. 26; new Supplementary Fig. 4**). These different cell types have varying proliferation abilities, resulting in organoids of different sizes. Initially, all organoids appear as round colonies when grow from single cells

(before day 10), and later form budding structures. Despite size differences, they ultimately achieve similar cell composition with high diversity, forming budding-structured organoids. Only a small number of organoids remain as round/cyst structures with lower cell diversity, likely due to the loss of stem cells (**Rebuttal Fig. 25b-c; new Supplementary Fig. 3d-e**).

Therefore, organoids in the TpC condition are mostly homogeneous in terms of cell composition and diversity, regardless of their sizes or shapes. Only a small number of organoids remain as round/cyst structures with lower diversity, representing true heterogeneity.



Rebuttal Fig. 26 TpC organoids originated from LGR5-positive and negative cells

3. The used ALPI staining makes it very hard to estimate how many enterocytes there really are in mature organoids compared to the secretory lineage (Figure 1 i,j,l. The enterocyte fraction is counted to be only 16.1% according to scRNAseq, which is low compared to the estimate in vivo abundance of ~70% (e.g., Wang et al., 2019). The bias towards secretory cells should be clearly noted in the manuscript.

Response:

Thank you for your question. We agree that there are fewer enterocytes compared to *in vivo* conditions, and this is due to the inherent design of the homogeneous organoid culture system, as detailed below:

1. Organoid culture maintains stem cells to maintain their expansion:

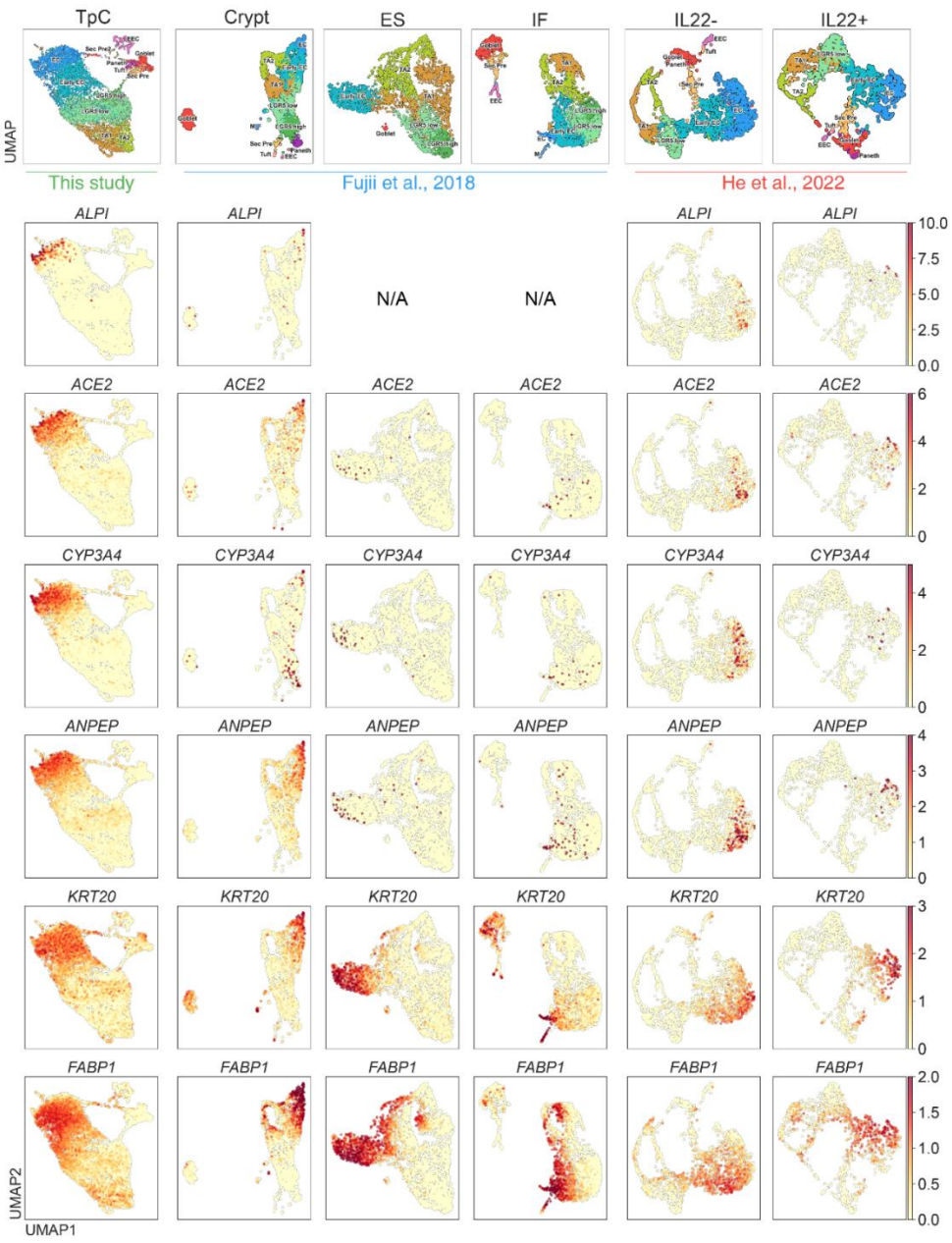
Indeed, there are fewer enterocytes in the organoids compared to *in vivo* conditions. As discussed in our response to major comment 6, a homogeneous organoid culture condition primarily aims to maintain and expand stem cells and progenitors, which is essential for organoid maintenance. This inherently affects the differentiation of terminally differentiated mature cells. The TpC condition strikes a balance between self-renewal and differentiation but cannot mimic the localized niche required for generating mature enterocytes as seen *in vivo*. This does not indicate a bias towards secretory cells; rather, EC lineage cells still represent a significant proportion of the organoid composition, albeit with reduced terminal differentiation.

2. Villus environment mimicking conditions promote increased enterocytes abundance:

We have provided an enterocyte differentiation protocol with high BMP and low Wnt signals to mimic niche signals at the villus. This significantly increased enterocyte abundance but at the cost of reduced organoid expansion potential.

3. TpC condition increased abundance of enterocytes over existing organoid protocols:

Our scRNA-seq analysis of the enterocyte cluster in human organoids shows that the lack of mature enterocytes is a common issue in human intestinal organoids. Previous conditions, such as the ES, IF (*Cell Stem Cell*, 2018), and IL22 (*Cell Stem Cell*, 2022) organoids, exhibit an almost complete absence of mature enterocytes (**Rebuttal Fig. 27**).



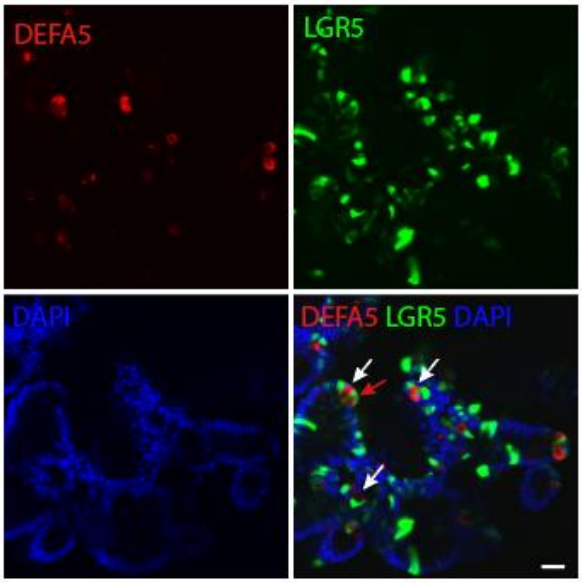
Rebuttal Fig. 27 scRNA-seq analysis of the enterocyte cluster

The observed lower abundance of enterocytes is not due to a bias towards secretory cells but rather a common limitation in organoid cultures that aim to balance stem cell maintenance and differentiation. And we believe that bioengineering approaches combined with our signal regulation methods could further increase the abundance of enterocytes and increase the fidelity of organoid systems.

4. Page 5, Line 21/22: The arrow does not point towards a double positive cell in Figure 1I as far as we can judge.

Response:

Thank you for pointing this out. We have updated Figure 1I (now **Fig. 1k** in the revised manuscript) to clearly show a double positive cell, indicated by a red arrow.



5. The authors show that a positive LGR5 cell can grow out to form a fully developed organoid. Is this true for other cell types as well?

Response:

Yes. We have added experiments showing that sorted LGR5-high, LGR5-low, and LGR5-negative cells are all able to generate new organoids (**Rebuttal Fig. 26; new Supplementary Fig. 4**). This suggests that, in addition to LGR5-positive cells, other cell types can also generate organoids. We have detailed this result in our response to reviewer 1, major comment 5.

6. The authors note that Figure 2g is only the secretory subset of the scRNAseq data. Why does it have EC, TA's and ISC's?

Response:

Thank you for pointing this out. As detailed in the "Clustering and Annotation" section of our Methods, we selected a subset of cells that includes secretory cells and their progenitors — ISCs, early enterocytes (ECs), and transient amplifying (TA) cells — for detailed analysis. This approach was taken to trace the progression from stem cells through different differentiation stages to secretory cells. Including ISCs, ECs, and TA cells allows us to capture the full differentiation pathway and lineage relationships. This is represented in Figure 2m, illustrating the complete trajectory within the secretory cell subset.

We have also updated the figure legend to clarify this:

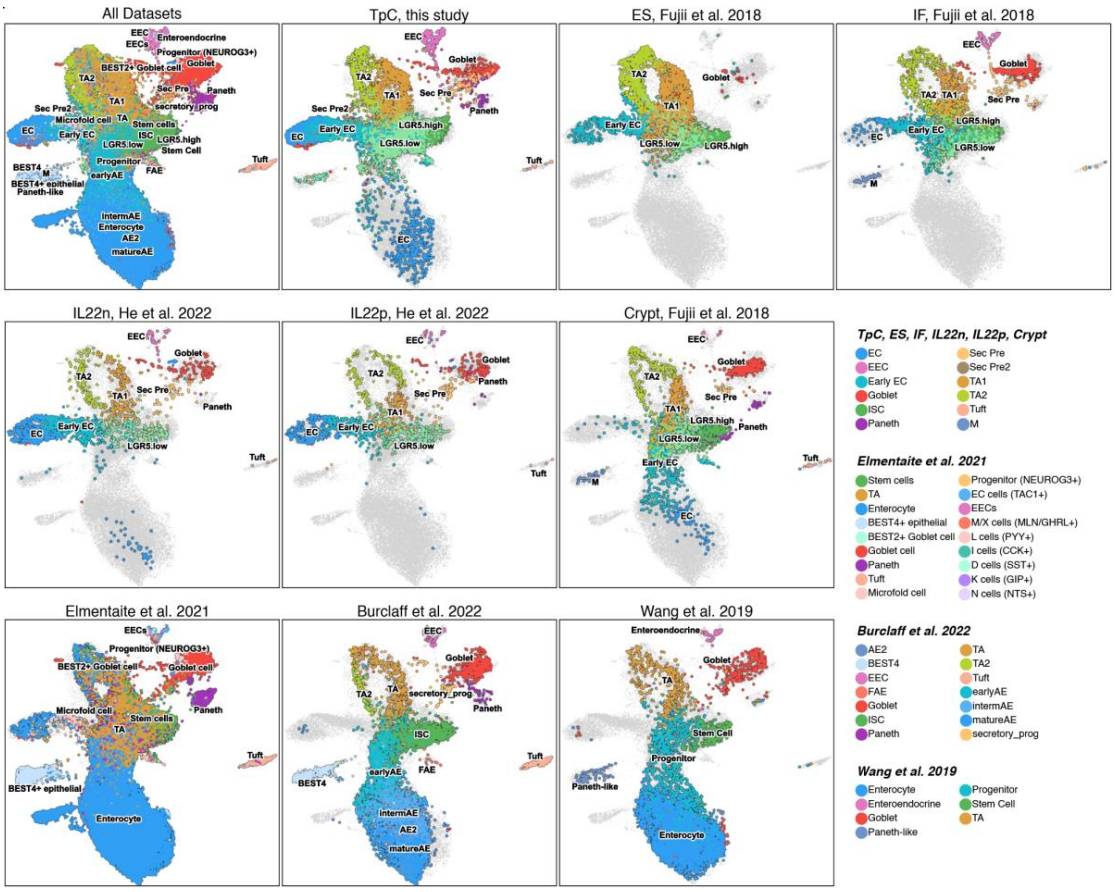
> *"Fig. 2g: UMAP plot showing subset analysis of secretory cells along with connected cell clusters."*

7. Can the authors argue on the mechanism of transdifferentiation observed from EC to GC? Is it caused by the medium, which may partially block EC differentiation? Chromatin landscape (see major point).

Response:

Thank you for this question. We hypothesize that the transdifferentiation from early enterocytes (EC) to goblet cells (GC) is influenced by the organoid culture medium, which is designed to expand progenitor cells and thus partially inhibits the terminal differentiation of mature cell types. For example, high Wnt and low BMP levels in the medium prevent the full differentiation of enterocytes.

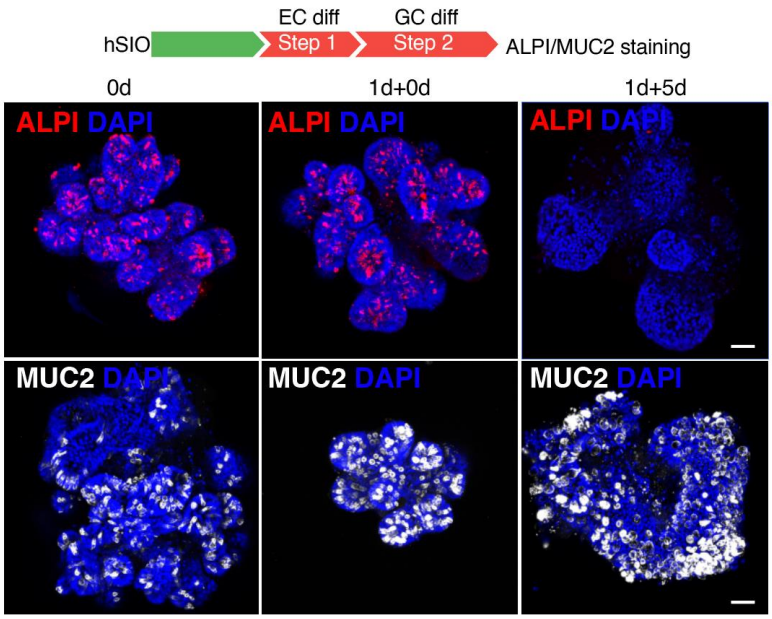
As supporting evidence, our single-cell RNA-seq analysis revealed that in current human organoid systems, including previous conditions (IF, ES, IL22 conditions), EC populations generally form a distinct cluster from *in vivo* EC lineage cells, despite similar marker gene expression (**Rebuttal Fig. 2B; new Supplementary Fig. 6b**).



Rebuttal Fig. 28 Harmony integration of single-cell RNA-seq datasets

In addition, although it is technically challenging to perform direct transdifferentiation experiments from pure enterocytes, we have added an experiment showing that partially differentiated enterocytes have the capacity to change their differentiation direction to goblet cells (**Rebuttal Fig. 29; new Supplementary Fig. 19**). As detailed in the revised manuscript:

> *"Trans-differentiation from early EC cells to goblet cells was implied from scRNA-seq data (Fig. 2l-m and Supplementary Fig. 5b). Similarly, when we transferred partially differentiated enterocytes into goblet cell differentiation conditions, we observed a decrease in enterocyte differentiation but maintained goblet cell differentiation (Supplementary Fig. 19). Although this experimental data is not sufficiently conclusive to fully support this speculation, it further suggests the plasticity of cells in the organoids."*

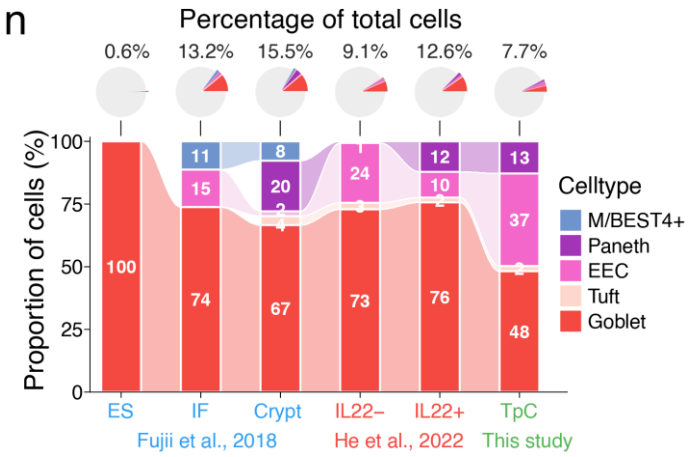


Rebuttal Fig. 29 Transdifferentiation of enterocytes lineage cells into goblet cells

8. Figure 2n is hard to read correctly. I would suggest to show piecharts as secretory % of the whole data set, instead of showing the same data which is underneath as a barchart.

Response:

Thank you for this comment. We have updated Fig. 2n following your suggestion.



9. Figure 3a/b indicates similar area but reduced LGR5 counts. This seems slightly contradicting. Can it be that the OLFM4 stem cell state is instead increased?

Response:

Thank you for this question. Yes, we have shown that LGR5-low cells, which have greater proliferation ability expresses higher OLFM4 than LGR5-high cells (Fig. 2d).

10. In many metrics (Figure 3), +pVc.CP seems almost equally good to TpC. Where is the advantage of TpC over it?

Response:

Thank you for this question. We use multiple metrics to evaluate our organoid system, including cell proliferation, the proportion of LGR5 cells, cellular diversity, colony-forming efficiency, and budding-structure formation. For these metrics, we have shown that:

Proportion of LGR5 Cells: The exclusion of TSA in +pVc.CP reduces the proportion of LGR5 cells, which are essential for generating Paneth cells and maintaining cellular diversity.

Cellular Diversity: TpC enhances the differentiation of multiple secretory cell types, particularly Paneth cells, resulting in greater cellular diversity.

Budding Formation: TpC promotes more robust budding-structure formation compared to +pVc.CP.

As per your suggestions in major comments 1 and 5, we have conducted additional experiments to clarify the effect and mechanism of TSA. These results are presented in **Figure 3a-h, Supplementary 10b-c and new Supplementary 12** in the revised manuscript and demonstrate the advantages of TpC in maintaining a higher proportion of LGR5 cells, greater cellular diversity, and improved budding formation.

We hope this addresses your concerns.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns, but this manuscript will not include some critical data (shown in the rebuttal letter). It is up to the editors to decide whether to revise the manuscript a second time in light of the data in rebuttal.

1. Rebuttal Fig.6: The authors demonstrated that LGR5-negative cells formed organoids more efficiently than LGR5-high ones in the TpC-conditioned medium. In response to this observation, they discussed how the TpC condition medium introduces the highly dynamic cell fate changes and plasticity of intestinal epithelial cells. It is very intriguing data, but unfortunately, the study suffers from major technical and an experimental design flaw. They need to show organoids can formed from LGR5+cells only using IF medium as a positive control. However, there might have never been any reports that non-stem cells can form organoids ex vivo, and I have no experience with that. There are some reports to identify robust stem cell markers in the human intestine and confirmed by performing organoid formation assay, LGR5 (Ishikawa et al. Gastroenterology 163(5), 2022), PTK7 (Jung et al. Stem Cell Rep 5(6), 2015), and EPHB2 (Jung et al. Nature Medicine 17(10), 2011). Those papers represented that human intestinal organoids are formed from only stem cell-positive cells. Therefore, they should explain the molecular mechanism of how organoids can be established from non-stem cells only when the TpC medium is used.

2. Rebuttal Fig.6: The authors discuss that the TpC medium introduces organoid formation derived from stem cell negative cells because it is a medium that promotes cell plasticity. However, Rebuttal Fig.5g indicates that organoid incubated in the TpC medium has more differentiated cells than in other mediums, which means the TpC medium is the medium that can efficiently induce differentiated cells. How do they explain this discrepancy?

3. The transdifferentiating experiment of Supplementary Fig.19 is problematic. From the general point of view of the organoid specialist, this short period is insufficient to induce differentiated cells, which means stem cells are still absolutely present in the organoids in the protocol. Therefore, it is possible that the MUC2-positive goblet cells observed at 1d+5d derived directly from stem cells but not enterocytes. The phenomenon of direct conversion of absorptive cells into secretory cells has not been reported not only in the intestine but also in other organs. Interpretation of scRNA-seq data alone is insufficient to explain this impactful biological event. Well-designed experiments should be used to prove it. The authors have the technology to make transgenic organoids that allow the isolate of only enterocytes (e.g., GFP knockin organoids in the ALPI gene locus by CRISPR/Cas9).

4. Response to my concern #7: I wondered why the stem cells decreased in this manuscript when the authors cultured organoid in +TSA (HDAC inhibitor) basal medium, which contains CHIR because a previous report indicated CHIR + VPA (another HDAC inhibitor) enhanced numbers of intestinal stem cell. In contrast, they claimed it is due to species specificity between mice and humans. However, their assertion is incorrect. The paper by Yin et al. (Nature Methods 2014, Supplementary Fig.3) represented that organoids are formed more efficiently in CHIR+VPA than in other conditions, not only in mice but also in humans.

Reviewer #2 (Remarks to the Author):

Dear Yang et al.,

Thank you for your comprehensive responses to our comments and your thoughtful revisions to the manuscript. We appreciate the effort you have put into addressing the concerns raised, and we believe that these changes have strengthened the work considerably. Below, we provide additional feedback based on your rebuttal and the revised manuscript.

Major points:

1. Chromatin-level analysis (e.g., ATAC-seq):

We understand that chromatin-level analysis was not within the original scope of your study. While we acknowledge your explanation, we believe that mapping the effects of the compounds on an epigenetic level would provide valuable insights into the mechanisms at play. This could be a fruitful avenue for future research, and we encourage you to consider such approaches in subsequent studies. Although it is not a current requirement, adding a brief mention of this as a limitation or a potential future direction in the discussion could strengthen the manuscript by outlining a clear path for future exploration.

2. Your rebuttal here is well-received. We are satisfied with your explanation and your recognition of other important studies in the field. The additional context you provided enriches the discussion and highlights your awareness of the broader landscape of research in this area.

3. References to human small Intestine only:

The focus on human small intestine markers is crucial for maintaining the manuscript's relevance and accuracy, and we are glad to see this adjustment was made. Please remove the references to colon-related studies, recent literature and dataset integrations show clear differences between colon and small intestine concerning the stem cell markers.

4. The corrections made to the labeling of EC cells and other textual refinements have enhanced the clarity of the manuscript. We commend your efforts in ensuring accuracy and clarity, as this will help prevent confusion in the field. Your proactive approach to refining the terminology and framework in this area is appreciated, and we believe the manuscript will contribute positively to ongoing research.

5. After reviewing the updated manuscript, we confirm that the revisions were carried out satisfactorily. The paper has benefitted from these changes and now reads more clearly and cohesively.

6. The final adjustments to the text are excellent. These have significantly improved the readability and scientific communication of your work, making it easier for other researchers in the field to follow your findings and conclusions.

General Questions:

Quantification of mNeonGreen intensity:

Could you clarify how mNeongreen signal has been quantified (e.g. per well or per segmented organoid)? And include it with the method section, please. We think it will enable future researchers to replicate your experiments accurately.

Cell density and Matrigel percentage:

For reproducibility purposes we would like to suggest emphasizing how many cells per microliter have been plated in a certain Matrigel percentage. Please include it in the methods section so that it stands out for future researchers looking to replicate your organoid culture conditions.

Conclusion:
Overall, we are very pleased with the revisions you have made in response to our comments. The manuscript is much improved and now provides a clearer contribution to the field, particularly regarding in vitro systems for the human small intestine. We believe that with these revisions, your work will be a valuable resource for future research, helping to advance the development of more physiologically relevant models.

We look forward to seeing this work published and believe it will be of great benefit to the scientific community.

Point-by-point response to comments of the reviewers

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns, but this manuscript will not include some critical data (shown in the rebuttal letter). It is up to the editors to decide whether to revise the manuscript a second time in light of the data in rebuttal.

1. Rebuttal Fig.6: The authors demonstrated that LGR5-negative cells formed organoids more efficiently than LGR5-high ones in the TpC-conditioned medium. In response to this observation, they discussed how the TpC condition medium introduces the highly dynamic cell fate changes and plasticity of intestinal epithelial cells. It is very intriguing data, but unfortunately, the study suffers from major technical and an experimental design flaw. They need to show organoids can formed from LGR5+cells only using IF medium as a positive control. However, there might have never been any reports that non-stem cells can form organoids ex vivo, and I have no experience with that. There are some reports to identify robust stem cell markers in the human intestine and confirmed by performing organoid formation assay, LGR5 (Ishikawa et al. Gastroenterology 163(5), 2022), PTK7 (Jung et al. Stem Cell Rep 5(6), 2015), and EPHB2 (Jung et al. Nature Medicine 17(10), 2011). Those papers represented that human intestinal organoids are formed from only stem cell-positive cells. Therefore, they should explain the molecular mechanism of how organoids can be established from non-stem cells only when the TpC medium is used.

Response:

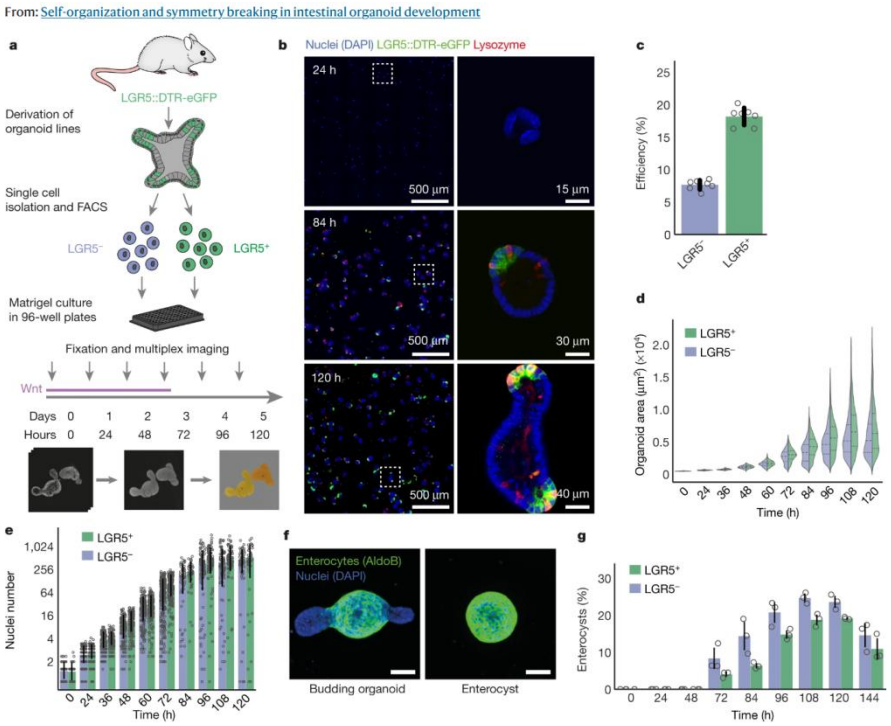
We appreciate your insightful comment and the opportunity to clarify our findings. We agree that the formation of organoids from LGR5-negative cells is a critical point that warrants detailed explanation. Below, we provide evidence from the literature and our own data to address this concern.

1. Lgr5-negative cells have the capability to form organoids

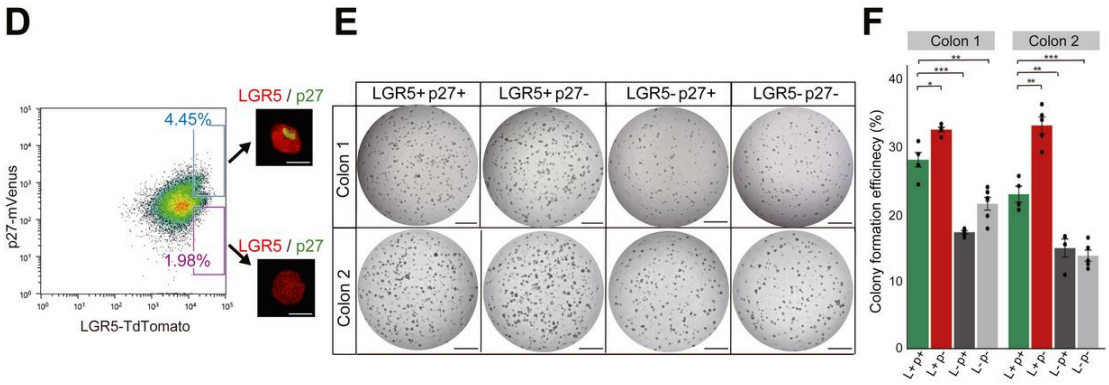
Several studies have demonstrated that LGR5-negative cells can form organoids in both mouse and human models:

- **Serra et al., Nature 2019:** This study showed that mouse Lgr5-negative cells formed organoids with an efficiency of approximately 7.5%, compared to 18% for Lgr5-positive cells (**Rebuttal Fig. 1c**).
- **Ishikawa et al., Gastroenterology 2022:** Human LGR5-negative cells have a colony-forming ability of about 20% (**Rebuttal Fig. 2F**).

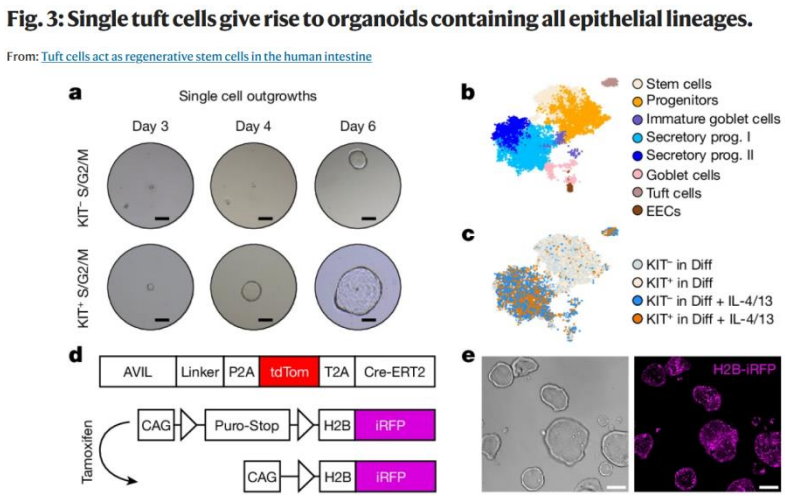
- **Huang et al., Nature 2024:** showed human LGR5 negative tuft cells could form organoids (**Rebuttal Fig. 3a**), although the exact efficiency was not specified.



Rebuttal Fig. 1. Serra et al., Nature 2019. Mouse Lgr5-negative cells form organoids.



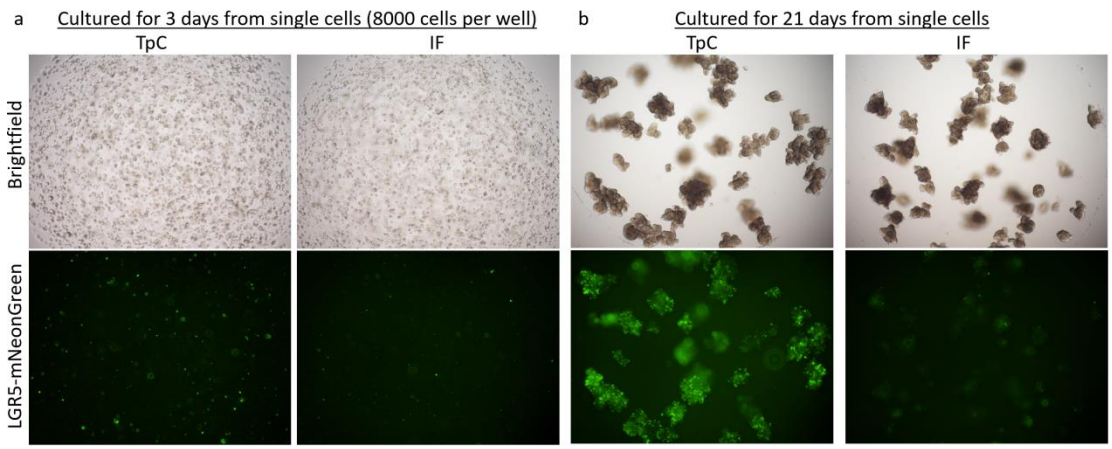
Rebuttal Fig. 2. Ishikawa K, et al. Gastroenterology, 2022. LGR5-negative cells have colony forming ability of about 20%.



Rebuttal Fig. 3. Huang et al., Nature 2024. LGR5 negative human tuft cells form organoids.

2. LGR5-negative cells in IF medium also can form organoids

In our own experiments, Under IF medium conditions, we observed minimal expression of LGR5-mNeonGreen, making it challenging to isolate LGR5-positive cells for organoid formation assays. Instead, we cultured mixed population of cells from the IF medium, containing both LGR5-positive and LGR5-negative cells. We observed that the colony formation efficiency exceeded the proportion of LGR5-mNeonGreen-positive cells (~ 1.5%) (**Rebuttal Fig. 4a**), indicating that LGR5-negative cells contribute to organoid formation even in IF medium. In our hands, over serial passages in IF medium, we noted a decrease in colony formation efficiency, and most organoids lacked high levels of LGR5-mNeonGreen expression (**Rebuttal Fig. 4b**). This suggests that IF medium maintains cells in a progenitor state but does not sustain high levels of LGR5 expression.



Rebuttal Fig. 4. Colony formation under TpC and IF medium

3. Non-Lgr5 cells may possess stemness or acquire it under certain conditions

LGR5-negative cells are not necessarily non-stem cells. Several studies have shown that various cell types within the intestinal epithelium possess stem cell potential or can acquire it under certain conditions. For example, transit-amplifying (TA) cells or isthmus progenitors have been shown to contribute to tissue regeneration (Malagola et al., Cell 2024; Capdevila et al., Cell 2024). Differentiated cell types, such as ALPI-positive enterocyte progenitors, can revert to a stem-like state during homeostasis or following injury (**Rebuttal Table 1**, from de Sousa e Melo and de Sauvage, Cell Stem Cell 2019).

Table 1. Summary of Intestinal Cells Capable of De-differentiation

Cell Type	Lineage	Damage	Suggested Mechanism of Plasticity	References
Bmi1/LRCs/EE	secretory and enteroendocrine progenitor	Lgr5-DTR - irradiation	open chromatin	Tian et al., 2011; Jadhav et al., 2017; Yan et al., 2017a, b
Dll1^{+}	secretory progenitor	irradiation	open chromatin-Atoh phosphorylation	Van Es et al., 2012a, b
TA	progenitor	helminth	Sca1-fetal conversion-Yap/Taz	Nusse et al., 2018
TA	progenitor	pathogens	Tuft-ILC2 circuit	von Moltke et al., 2016; Schneider et al., 2018
Paneth	secretory	Lgr5-DTR-DSS	Notch-SCF/ckit1	Schmitt et al., 2018; Yu et al., 2018
Alpi^{+}	enterocyte progenitor	Lgr5-DTR	Fth1- Proliferation-Chromatin status	Tetteh et al., 2016
Krt19^{+}	upper crypt progenitor	Irradiation-5FU	unknown	Asfaha et al., 2015

Rebuttal Table 1. From Felipe de Sousa e Melo and Frederic J. de Sauvage's review. Cell Stem cell (2019).

4. Culture conditions affect organoid formation

Our understanding is that the ability of cells to form organoids is influenced by both intrinsic properties and extrinsic factors provided by the culture environment:

LGR5-positive stem cells typically rely on niche signals provided by Paneth cells in vivo. In vitro, the ability of cells to generate organoids depends on recreating this niche or providing the necessary signals exogenously. The TpC medium contains additional niche factors, such as Wnt and possibly Notch, which can support the survival and proliferation of LGR5-negative cells. This enriched environment may enable LGR5-negative cells to either maintain their latent stemness or acquire stem cell properties necessary for organoid formation. In contrast, the IF medium may lack sufficient niche signals to support organoid formation from LGR5-negative cells to the same extent. This may explain why organoid formation efficiency decreases over serial passages in IF medium

and why organoids formed may lack high LGR5 expression.

We believe that these findings address your concerns and provide a mechanistic explanation for how organoids can be established from non-stem (LGR5-negative) cells when the TpC medium is used.

Reference:

1. *Felipe de Sousa e Melo, Frederic J. de Sauvage, Cellular Plasticity in Intestinal Homeostasis and Disease. Cell Stem Cell 24, 54-64 (2019)*
2. *Tetteh, P., et al. Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters. Cell Stem Cell 18, 203-213 (2016)*
3. *Serra, D., et al. Self-organization and symmetry breaking in intestinal organoid development. Nature 569, 66-72 (2019).*
4. *Ishikawa, K., et al. Identification of Quiescent LGR5+ Stem Cells in the Human Colon. Gastroenterology 163,1391-1406 (2022).*
5. *Malagola, E., et al. Isthmus progenitor cells contribute to homeostatic cellular turnover and support regeneration following intestinal injury. Cell 187, 3056-3071 e3017 (2024).*
6. *Capdevila C., et al. Time-resolved fate mapping identifies the intestinal upper crypt zone as an origin of Lgr5+crypt base columnar cells. Cell 187, 3039-3055.e14 (2024).*

2. Rebuttal Fig.6: The authors discuss that the TpC medium introduces organoid formation derived from stem cell negative cells because it is a medium that promotes cell plasticity. However, Rebuttal Fig.5g indicates that organoid incubated in the TpC medium has more differentiated cells than in other mediums, which means the TpC medium is the medium that can efficiently induce differentiated cells. How do they explain this discrepancy?

Response:

Thank you for your comment regarding the effects of the TpC media on organoid formation and differentiation.

We understand that it may seem contradictory that the TpC medium, which promotes cell plasticity, results in organoids with more differentiated cells. However, this outcome

aligns with our main hypothesis and strategy in the study: to increases cellular diversity through enhancing cellular plasticity in human organoids.

By promoting stem cell plasticity, the TpC medium expands the pool of stem cells capable of generating various specialized cell types. This results in organoids with both an expanded stem cell compartment and a higher number and diversity of differentiated cells, especially Paneth cells, achieving a cellular composition that more closely mirrors native intestinal tissue.

Therefore, the increase in differentiated cells is a direct outcome of the enhanced stem cell plasticity induced by the TpC medium. We hope this clarifies how the TpC medium contributes to both stem cell maintenance and differentiation, addressing the apparent discrepancy.

3. The transdifferentiating experiment of Supplementary Fig.19 is problematic. From the general point of view of the organoid specialist, this short period is insufficient to induce differentiated cells, which means stem cells are still absolutely present in the organoids in the protocol. Therefore, it is possible that the MUC2-positive goblet cells observed at 1d+5d derived directly from stem cells but not enterocytes. The phenomenon of direct conversion of absorptive cells into secretory cells has not been reported not only in the intestine but also in other organs. Interpretation of scRNA-seq data alone is insufficient to explain this impactful biological event. Well-designed experiments should be used to prove it. The authors have the technology to make transgenic organoids that allow the isolate of only enterocytes (e.g., GFP knockin organoids in the ALPI gene locus by CRISPR/Cas9).

Response:

Thank you for your insightful comment regarding the transdifferentiation experiment presented in Supplementary Fig. 19. We acknowledge that the experiment in Supplementary Fig. 19 may not provide definitive proof of direct conversion from enterocytes to goblet cells, as stem cells might still be present during the short period.

In our experiment, we observed not only the formation of goblet cells but also the loss of ALP expression when cells were exposed to goblet cell differentiation conditions. This suggests plasticity in the ALPI+ cells generated at day 1 of differentiation. Prior research has demonstrated that ALPI+ absorptive cells can dedifferentiate into stem cells in vivo (Tetteh et al., Cell Stem Cell 2016), supporting the idea that these cells possess inherent plasticity. Thus, while the data may not confirm direct transdifferentiation, it indicates the

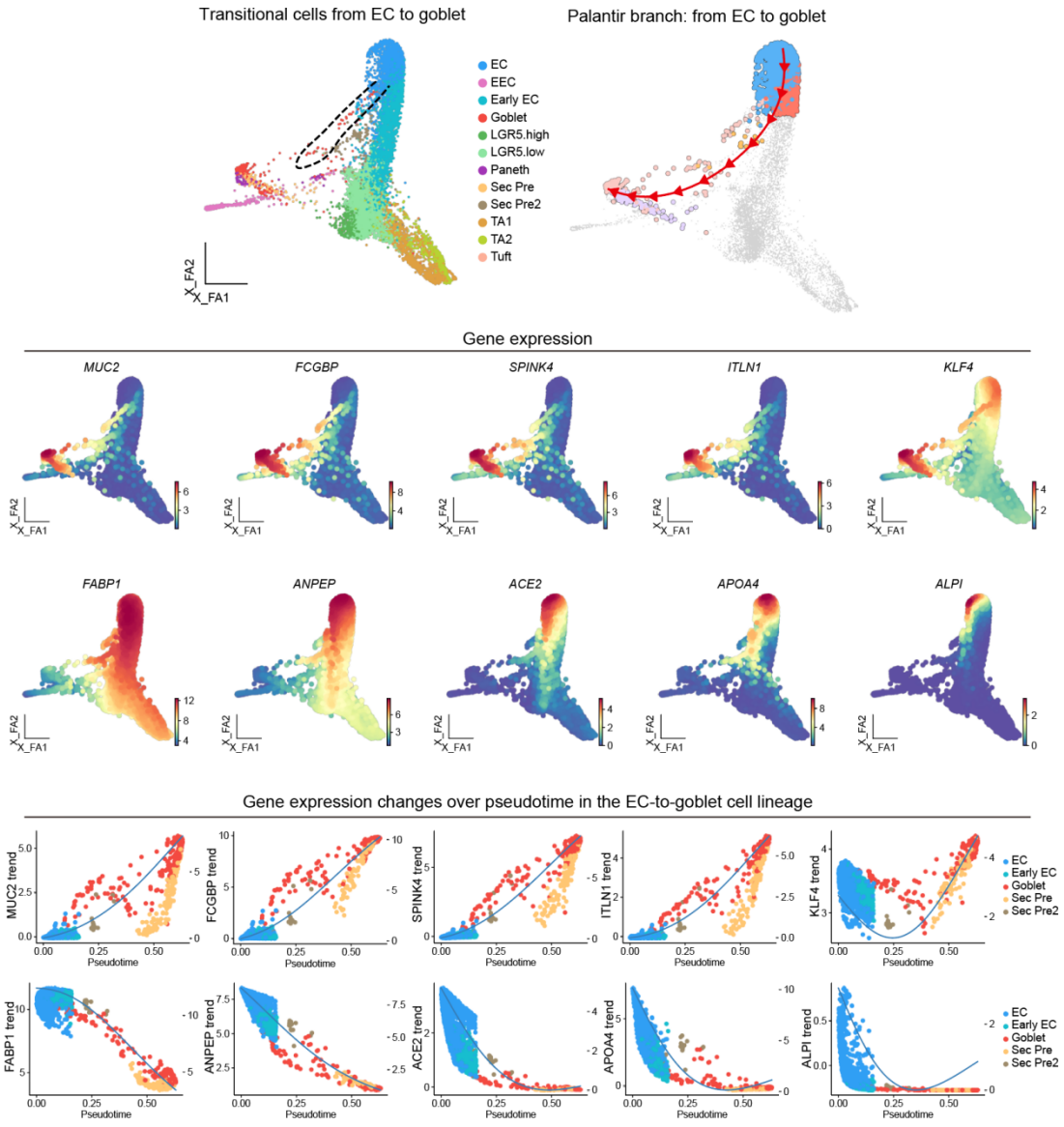
potential for lineage flexibility within the organoid system.

To strengthen the findings from scRNA-seq, we re-analyzed the data using diffusion maps and trajectory analysis tools, such as Palantir. This analysis revealed trajectories starting from enterocyte cell populations, capturing intermediate states and indicating potential cell fate changes. This suggests a transition between enterocyte marker-expressing cells and goblet cell-expressing cells in our dataset (**Rebuttal Fig. 5**). From this in-depth analysis of the scRNA-seq data, we believe that that ALPI may not be the most effective marker to track goblet cell formation from absorptive cells. Alternative markers like ACE2, APOA4, or ANPEP could better capture transdifferentiation events. We also hypothesize that during the differentiation of stem cells into absorptive cells, their ability to generate secretory cells gradually diminishes, with partially differentiated cells possibly retaining varying transdifferentiation potential.

However, we agree that conclusively demonstrating direct transdifferentiation requires additional well-designed experiments, such as lineage tracing using transgenic organoids. We plan to perform these studies in future research, utilizing advanced technologies like unbiased cell barcoding, lineage-tracing techniques, and CRISPR-mediated knock-in of reporters to confirm these transitions. These approaches will help us gain a more comprehensive understanding of the plasticity and dynamics of human intestinal cell fate transitions, including differentiation, self-renewal, transdifferentiation, and dedifferentiation.

In light of these limitations and to avoid any potential misinterpretation, we have decided to remove Supplementary Fig. 19 from the manuscript. We believe this strengthens the manuscript’s conclusions and allows us to focus on the core findings, while also providing an exciting direction for future research into cell plasticity and fate transitions in human organoid systems.

We appreciate your thoughtful feedback, which has significantly contributed to improving the clarity and rigor of our study.



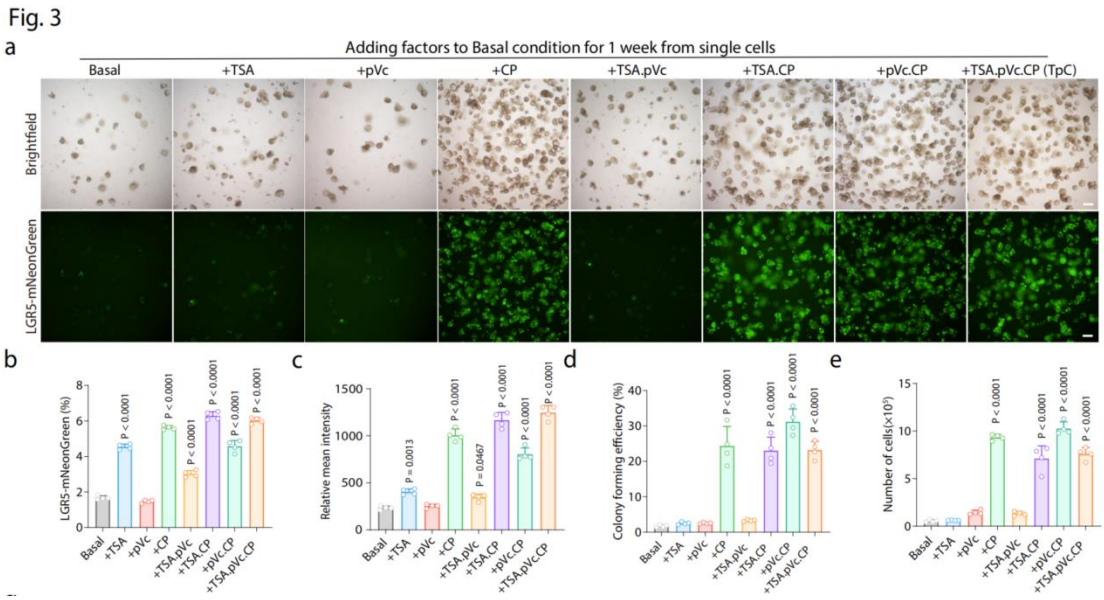
Rebuttal Fig. 5. Reanalysis of scRNA-seq data for EC to goblet transition

4. Response to my concern #7: I wondered why the stem cells decreased in this manuscript when the authors cultured organoid in +TSA (HDAC inhibitor) basal medium, which contains CHIR because a previous report indicated CHIR + VPA (another HDAC inhibitor) enhanced numbers of intestinal stem cell. In contrast, they claimed it is due to species specificity between mice and humans. However, their assertion is incorrect. The paper by Yin et al. (Nature Methods 2014, Supplementary Fig.3) represented that organoids are formed more efficiently in CHIR+VPA than in other conditions, not only in mice but also in humans.

Response:

Thank you for your thoughtful comment and highlight this important point.

We would like to clarify that in our manuscript, we observed that adding TSA (an HDAC inhibitor) to the basal medium (which contains CHIR) **increases** the number of stem cells compared to the basal medium alone. This is demonstrated in our original as well as previously revised manuscript (**Fig. 3a-c, Rebuttal Fig. 6**), where the addition of TSA enhances stem cell numbers in human organoids. Our findings are **consistent** with the results reported in Yin et al. (Nature Methods 2014, Supplementary Fig. 3), where we showed that CHIR combined with VPA (another HDAC inhibitor) enhanced organoid formation efficiency in both mouse and human intestinal organoids.



Rebuttal Fig. 6: TSA increases stem cell numbers in organoids when added to the basal medium (the original Fig. 3a-c in our previous revised manuscript).

We would like to clarify the comparison between the effectiveness of **Basal + TSA** and the **TpC medium**. While TSA does enhance stem cell numbers compared to the basal medium, it is **not as effective** as the TpC medium in maintaining and expanding stem cells in human organoids. This is why in our manuscript, we discussed species-specific differences between mice and humans regarding the sufficiency of CHIR and HDAC inhibitors:

- **In mouse organoids**, the combination of CHIR and an HDAC inhibitor (like VPA) is **sufficient** to maintain stem cells.
- **In human organoids**, this combination **is effective**, but **not sufficient** and additional factors provided in the TpC medium are necessary to promote optimal

cell proliferation and colony formation.

Our statement about species specificity was meant to explain that, although CHIR + HDAC inhibitors can enhance stem cell numbers in both species, the degree of effectiveness and the requirement for additional factors differ between mice and humans. The TpC medium contains additional components (e.g., Cp) that are crucial for human stem cell maintenance and organoid formation, which are not required to the same extent in mouse organoids.

We apologize for any confusion our previous explanation may have caused and appreciate the opportunity to clarify this point. We hope this addresses your concern and aligns our findings with those reported previously in Yin et al.

Reviewer #2 (Remarks to the Author):

Dear Yang et al.,

Thank you for your comprehensive responses to our comments and your thoughtful revisions to the manuscript. We appreciate the effort you have put into addressing the concerns raised, and we believe that these changes have strengthened the work considerably. Below, we provide additional feedback based on your rebuttal and the revised manuscript.

Response:

We thank you for your positive feedback and are pleased that our revisions have strengthened the manuscript. We appreciate your help in strengthen our work.

Major points:

1. Chromatin-level analysis (e.g., ATAC-seq):

We understand that chromatin-level analysis was not within the original scope of your study. While we acknowledge your explanation, we believe that mapping the effects of the compounds on an epigenetic level would provide valuable insights into the mechanisms at play. This could be a fruitful avenue for future research, and we encourage you to consider such approaches in subsequent studies. Although it is not a current requirement, adding a brief mention of this as a limitation or a potential future direction in the discussion could strengthen the manuscript by outlining a clear path for future exploration.

Response:

Thank you for this valuable and constructive suggestion. We agree that employing technologies like ATAC-seq to explore the effects and mechanisms of the compounds at the epigenetic level would provide significant insights. We plan to include such analyses in our future research. To acknowledge this important point, we have added it to the discussion section as a potential direction for future exploration.

"Further investigation into the epigenetic mechanisms of HDAC inhibitors, using tools such as ATAC-seq, would provide valuable insights and represents a promising direction for future research."

2. Your rebuttal here is well-received. We are satisfied with your explanation and your recognition of other important studies in the field. The additional context you provided enriches the discussion and highlights your awareness of the broader landscape of research in this area.

Response:

Thank you for this positive feedback.

3. References to human small Intestine only:

The focus on human small intestine markers is crucial for maintaining the manuscript's relevance and accuracy, and we are glad to see this adjustment was made. Please remove the references to colon-related studies, recent literature and dataset integrations show clear differences between colon and small intestine concerning the stem cell markers.

Response:

Thank you for your suggestion. We have removed the references of colon-related studies.

4. The corrections made to the labeling of EC cells and other textual refinements have enhanced the clarity of the manuscript. We commend your efforts in ensuring accuracy and clarity, as this will help prevent confusion in the field. Your proactive approach to refining the terminology and framework in this area is appreciated, and we believe the manuscript will contribute positively to ongoing research.

Response:

We appreciate your positive feedback.

5. After reviewing the updated manuscript, we confirm that the revisions were carried out satisfactorily. The paper has benefitted from these changes and now reads more clearly and cohesively.

Response:

We appreciate your positive feedback.

6. The final adjustments to the text are excellent. These have significantly improved the readability and scientific communication of your work, making it easier for other researchers in the field to follow your findings and conclusions.

Response:

Thank you for your encouraging feedback.

General Questions:

Quantification of mNeonGreen intensity:

Could you clarify how mNeongreen signal has been quantified (e.g. per well or per segmented organoid)? And include it with the method section, please. We think it will enable future researchers to replicate your experiments accurately.

Response:

We measured the mNeonGreen signal at the single-cell level using flow cytometry (FACS). Organoids from one or more wells were collected, dissociated into single cells, and analyzed by FACS without segmenting individual organoids. This allowed us to quantify mNeonGreen fluorescence per cell across the organoid population.

We have updated the Methods section to include these details, ensuring that future researchers can replicate our experiments accurately.

Cell density and Matrigel percentage:

For reproducibility purposes we would like to suggest emphasizing how many cells per microliter have been plated in a certain Matrigel percentage. Please include it in the methods section so that it stands out for future researchers looking to replicate your organoid culture conditions.

Response:

Thank you for your suggestion. In our organoid culture conditions:

Matrigel Percentage: We used Matrigel pre-diluted with Advanced DMEM/F12 at a ratio of 5:3 (Matrigel to Advanced DMEM/F12, v/v).

Cell Density: Cells were suspended in the Matrigel mixture at a density of approximately 8,000 cells per 20 microliters, which equates to 400 cells per microliter.

Plating Details: We plated 20 microliters of the cell-Matrigel mixture per well in a 48-well plate.

We have included these details in the Methods section of the revised manuscript to ensure that future researchers can accurately replicate our organoid culture conditions.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

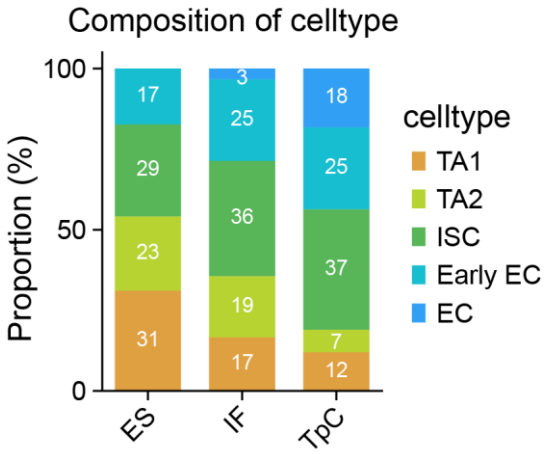
I think the authors have answered the critiques satisfactorily, and your rebuttal comments here are well-received. But the one concern remains unresolved. Against my concern #2, the authors explained that the TpC medium simultaneously increases both the intestinal stem cell compartment and the number of differentiated cells by expanding the stem cell pool. Actually, they demonstrated that the "percentages" (not cell numbers) of intestinal stem cells, but also all lineage cells of the intestinal epithelium, were increased in Rebuttal Fig. 5. However, there must be a rise in some cell types and a fall in others if they analyzed the percentages of cell types that comprise the organoids. Why is there no cell type at all that is decreasing its proportion among the cells that make up the organoids? The authors should provide a clear explanation for that.

Response:

We appreciate your continued scrutiny and feedback. Regarding your concern about cell type proportions in the organoids, our hypothesis is that the TpC condition enhances cell plasticity by converting intermediate progenitors into stem cells, which then generate a more diverse population of differentiated cells, resulting in a decrease in progenitor cell proportions.

While we previously highlighted the increase in secretory lineages (Paneth, goblet, and enteroendocrine cells) and stem cells under TpC conditions compared to IF conditions, we have now analyzed other major cell populations. Although direct comparison of cell proportions between different datasets has inherent limitations due to variations in culture duration, sample collection approaches, and batch effects (as discussed in our previous responses), our analysis reveals that transit-amplifying (TA) cells show a decreased proportion under TpC conditions compared to both ES and IF conditions (**Rebuttal Fig. 1**).

This reduction in TA cells, alongside increases in both stem cells and differentiated cells, aligns with our hypothesis that TpC condition promotes cell plasticity by facilitating the conversion of intermediate progenitors into stem cells, which subsequently differentiate into various terminal cell types. We hope this addresses your concern.



Rebuttal Fig. 1. Comparison of cell type proportions across ES, IF, and TpC culture conditions