

**Reviews for** “Human pluripotent stem cell-derived organoids as a model of intestinal xenobiotic metabolism”

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**Decision:** Revise and resubmit pending major revisions

**Reviewer 1** has selected to remain anonymous.

Originality, novelty and significance of results: Inadequate

Technical Quality of Work: Inadequate

Comprehensibility and Presentation of Paper: Adequate

What is the overall impression: Inadequate

**Reviewer Recommendation Term:** Revise and resubmit pending major revisions

**Narrative (as sent to corresponding author):**

Although the writing of the article is adequate, the article makes a lot of claims that would require more extensive study than what is presented. One of the major claims of the article, that the organoids generated have epithelium that faces outward, is in contradiction to their previously published work in JCI Insight. In that article they performed forskolin swelling assays to show that organoids have CFTR activity. This assay is dependent on the epithelium facing inward so that sodium is pumped into the lumen creating an inbalance in sodium which is quickly offset by water rushing into the lumen creating a swelling effect. Figure 1 lacks any quantitation. For instance, what percentage of epithelial cells actually express CHGA or CDX2? More extensive quantitation on multiple organoids is needed. I would also include Ezrin (an apical marker) staining to show that parts of the epithelium face outward.
Figure 2 lacks a negative control. In addition, since the methods only mention one small intestine sample, I have to guess that the values presented are technical replicates of the same sample. In the text the authors also mention that (line 204) a single organoid was used for qPCR analysis. If this is the case I don't see how they can run statistics considering that they have 1 biological replicate. I also don't understand why the authors didn't simply run an RNA seq analysis. This figure requires at least 3 different small intestinal RNA samples and 3 different organoids as well as a negative control (pluripotent stem cells).
Figure 3 is adequate except that there should be more than 1 intestine sample.
Figure 4 does not have the optimal control. Either an inhibitor of CYP3A4 needs to be used to show specific CYP3A4 activity or organoids which lack CYP3A4 or have CYP3A4 knockdown (shRNA) should be used.
Supplementary figure 3 does not have a negative controls for staining.

**Reviewer 2** has selected to remain anonymous.

Originality, novelty and significance of results: Adequate

Technical Quality of Work: Adequate

Comprehensibility and Presentation of Paper: Good

What is the overall impression: Adequate

**Reviewer Recommendation Term:** Revise and resubmit pending major revisions

**Narrative (as sent to corresponding author):**

In their paper entitled "Human pluripotent stem cell-derived organoids as a model of intestinal xenobiotic metabolism", Sasaki et al. use human pluripotent stem cell-derived organoids generated in xenogeneic-free conditions, a method previously published by this group, to examine the expression, induction, and activity of key drug transporter/metabolism genes and proteins. They find that many of these genes are expressed at similar levels between their XF organoids and human intestinal tissue. They can further induce the expression of CYP3A4 and ABCB1 following the addition of Vitamin D. They use a luciferase-based assay to demonstrate CYP3A4 activity. This manuscript would benefit from additional antibody reagent validation, analysis of epithelial barrier function (polarity, junctions, brush border, permeability), and additional controls for the CYP3A4 activity assay. In addition, further discussion to compare and contrast this XF organoid model with other organoids models for pharmacological testing would provide better context for this study within the field. Overall, it is important to continue to pursue the development of suitable in vitro models that could be used to assess drug metabolism and toxicity, so this study will be of interest to the readership of Stem Journal.

Major comments:
1. It will be important for the authors to do a more thorough comparison of their study to previous studies published in the literature that present intestinal organoid models for preclinical drug studies. A quick search identified at least a couple (Kasendra et al. Elife 2020 PMID: 31933478; Onozato et al. Drug Metab Dispos 2018 PMID: 29615438), and the authors already cited another one by Madden et al., although there might be more. What are the advantages or disadvantages of the model presented here?

2. Immunostaining of human intestinal tissue for CES1 and CES2 in Supplementary Fig. S3 is an important positive control. This type of positive control (and suitable negative controls) should be shown for the other antibodies used in Fig. 2 of this study, SLC10A2, SLC15A1, and CYP3A4.
SLC10A2 and SLC15A1 antibodies typically stain specifically at the brush border of intestinal tissue, but this pattern is not observed in Fig. 2 organoids. These antibodies must be validated. If they can be validated and still do not follow this expected staining pattern, then is the brush border mature in these organoids? I can't see the labels for the bottom 3 panels of immunostaining (the labels are cut off in the pdf file), but I'm assuming that they are in the same order listed in the figure legend, and if so, then the CYP3A4 staining is not convincing and appears to be mostly nuclear rather than cytoplasmic. This antibody also should be validated.

3. The authors state several times in their manuscript that their organoids are outward oriented, but the images provided do not definitively show this. Also, no epithelial polarity or junction markers are shown to demonstrate orientation.

4. The CYP3A4 activity luciferase approach used in this study appears to have been based on the assumption that the organoids have a functional barrier. Organoid permeability data should be included. In addition, the CYP3A4 activity data is not very convincing as shown. One important control would be data from XF organoids not exposed to the proluciferin substrate. In addition, because the authors are proposing that this culture system would be suitable for drug assessment, it would be important to show that the CYP3A4 activity could be modulated (up- and/or down-regulated) in response to a treatment condition.

**AUTHOR’S REPLY TO REVIEWERS**

Point-by-point response to the Reviewers:

We greatly appreciate the careful reading of the manuscript and the constructive suggestions made by the Reviewer; we have used these comments and criticisms to improve our study and the manuscript. Below, we address the comments and provide our response to each of these.

Reviewer #1 (Remarks to the Author):
Although the writing of the article is adequate, the article makes a lot of claims that would require more extensive study than what is presented. One of the major claims of the article, that the organoids generated have epithelium that faces outward, is in contradiction to their previously published work in JCI Insight. In that article they performed forskolin swelling assays to show that organoids have CFTR activity. This assay is dependent on the epithelium
facing inward so that sodium is pumped into the lumen creating an inbalance in sodium which is quickly offset by water rushing into the lumen creating a swelling effect.
Response
We are grateful to the reviewer for the critical comments and useful suggestions that have helped us to improve our paper. As indicated in the responses that follow, we have taken all these comments and suggestions into account in the revised manuscript.

[Comments of the reviewer #1]
1. Figure 1 lacks any quantitation. For instance, what percentage of epithelial cells actually express CHGA or CDX2? More extensive quantitation on multiple organoids is needed. I would also include Ezrin (an apical marker) staining to show that parts of the epithelium face outward.

Action/Response
Thank you for pointing these out. CDX2 and CHGA positive cells in the epithelium of XF-HIOs was 95.3% or 10.6%, respectively. The data was newly added in the revised manuscript as Supplementary Figure 1a and b.
We may lack some explanations on the epithelial layers facing outward. Figure 1b, 1d and 2c show that the epithelium layers of XF-HIOs face outwards. We also added Ezrin staining as Supplementary Figure 1c. Ezrin positively localized to an apical part of the epithelial layers in the XF-HIOs.

2. Figure 2 lacks a negative control. In addition, since the methods only mention one small intestine sample, I have to guess that the values presented are technical replicates of the same sample. In the text the authors also mention that (line 204) a single organoid was used for qPCR analysis. If this is the case I don't see how they can run statistics considering that they have 1 biological replicate. I also don't understand why the authors didn't simply run an RNA seq analysis. This figure requires at least 3 different small intestinal RNA samples and 3 different organoids as well as a negative control (pluripotent stem cells).

Action/Response
We thank the reviewer for pointing these out and apologize for the confusion; we had missed to specify n=5 for the number of biological samples in the body text, XF-HIOs in Fig 2a and b. The qRT-PCR data in question was generated from an experiment with 5 independent organoids, and each organoid (“a single organoid” in use) analyzed with qRT-PCR. All qRT-PCR analysis in Fig 2 (also in Supplementary Fig 2 and 3) were performed by Qiagen RT2 Profiler PCR Arrays. This data was averaged to produce the graph data in Figure 2. We added the explanation on the qRT-PCR analysis in the Materials and Methods.
We increased the number of small intestinal RNA samples to 3 samples and ran qRT-PCR independently on all 3 of the samples. The results from this qRT-PCR were averaged and set to 1.0 as the reference sample. We reanalyzed the qRT-PCR with additional control intestines and human ES cells (new Fig 2a and b, and Supplementary Fig 2 and 3). The results of the manuscript are not affected by the recanalization.

3. Figure 3 is adequate except that there should be more than 1 intestine sample.

Action/Response
We added two more small intestinal RNA samples to 3 samples in Figure 3.

4. Figure 4 does not have the optimal control. Either an inhibitor of CYP3A4 needs to be used to show specific CYP3A4 activity or organoids which lack CYP3A4 or have CYP3A4 knockdown (shRNA) should be used.

Action/Response
We thank the reviewer for pointing this out. We freshly re-performed CYP3A4 catalytic assays and included CYP3A4 inhibition assay using Ketoconazole (KCZ), which the Food and Drug Administration (FDA) and European Medicines Agency (EMA) recommended as a strong CYP3A4 inhibitor in clinical drug-drug interaction (DDI) studies (new Figure 4). The results of the manuscript is not affected by the recanalization, but our statistically analysis in cont. vs IPA and IPA vs IPA+KCZ showed p = 0.05057 and P = 0.06438, respectively. In this result, statistically higher phrase was changed.

Supplementary figure 3 does not have a negative controls for staining.

Action/Response
We added negative controls of CES1 and CES2 in Supplementary figure 4.
Reviewer #2 (Remarks to the Author):
In their paper entitled "Human pluripotent stem cell-derived organoids as a model of intestinal xenobiotic metabolism", Sasaki et al. use human pluripotent stem cell-derived organoids generated in xenogeneic-free conditions, a method previously published by this group, to examine the expression, induction, and activity of key drug transporter/metabolism genes and proteins. They find that many of these genes are expressed at similar levels between their XF organoids and human intestinal tissue. They can further induce the expression of CYP3A4 and ABCB1 following the addition of Vitamin D. They use a luciferase-based assay to demonstrate CYP3A4 activity. This manuscript would benefit from additional antibody reagent validation, analysis of epithelial barrier function (polarity, junctions, brush border, permeability), and additional controls for the CYP3A4 activity assay. In addition, further discussion to compare and contrast this XF organoid model with other organoids models for pharmacological testing would provide better context for this study within the field. Overall, it is important to continue to pursue the development of suitable in vitro models that could be used to assess drug metabolism and toxicity, so this study will be of interest to the readership of Stem Journal.
Response
We are grateful for the reviewer’s positive views and insightful comments.

[Comments of the reviewer]
1. It will be important for the authors to do a more thorough comparison of their study to previous studies published in the literature that present intestinal organoid models for preclinical drug studies. A quick search identified at least a couple (Kasendra et al. Elife 2020 PMID: 31933478; Onozato et al. Drug Metab Dispos 2018 PMID: 29615438), and the authors
already cited another one by Madden et al., although there might be more. What are the advantages or disadvantages of the model presented here?

Action/Response
Thank you for the critical comments. So far, some intestinal epithelium models have been reported, and a couple of them have examined on gene expressions involved in drug metabolism. However, most of them including Kasendra et al. Elife 2020, showed gene expression status related with drug metabolism, so metabolic functionality of the xenobiotics in human intestinal in vitro models still remains elusive. We provided a total 170 genes expression status (86 transporters and 84 metabolizing enzymes) by quantitative RT-PCR, and also showed metabolic activity of a key protein, CYP3A4 in this manuscript.
Human tissue derived models including Kasendra et al. Elife 2020, are necessary for use of “real tissue” by tissue biopsy. There could be significant challenges for Lot control (each product quality and availability, etc) in general, same confronting issues as use in primary human hepatocytes. Another models of 3D intestinal organoids (or enteroids) including from PSCs (e.c. Onozato et al. Drug Metab Dispos 2018) or biopsy specimens, restricts access to its lumen due to their apical-inn architecture, which is crucial for assessing epithelial permeability or drug absorption. Furthermore, their 3D organoids are much smaller size comparing with our XF-HIOs, so none of reports showed a single of organoid can partialize and metabolize xenobiotics. In the manuscript we presented that a single XF-HIO can perform absorption and metabolization on xenobiotics. Generally, “the characterization of the pharmacokinetic properties of 3D organoids, as well as the validation for its use in drug discovery and development, is still very limited” (Elife 2020). We hope our study would enhance to explore the potential of the pharmacokinetic properties of gastrointestinal organoids.
We have reported a review article which pointed out comparisons (biological characteristics and advantages/limitations in use) among epithelial organoids, HIOs and XF-HIOs (Tsuruta S, et al. JMA Journal, 2020, cited #8 in this manuscript).

2. Immunostaining of human intestinal tissue for CES1 and CES2 in Supplementary Fig. S3 is an important positive control. This type of positive control (and suitable negative controls) should be shown for the other antibodies used in Fig.2 of this study, SLC10A2, SLC15A1, and CYP3A4. SLC10A2 and SLC15A1 antibodies typically stain specifically at the brush border of intestinal tissue, but this pattern is not observed in Fig. 2 organoids. These antibodies must be validated. If they can be validated and still do not follow this expected staining pattern, then is the brush border mature in these organoids? I can't see the labels for the bottom 3 panels of immunostaining (the labels are cut off in the pdf file), but I'm assuming that they are in the same order listed in the figure legend, and if so, then the CYP3A4 staining is not convincing and appears to be mostly nuclear rather than cytoplasmic. This antibody also should be validated.

Action/Response
We are grateful for the reviewer’s comments. We apologize for the cut-off of Figure 2C in the PDF file. We performed immunostaining of additional XF-HIOs for SLC10A2 and CYP3A4. In the revised manuscript, SLC10A2 and CYP3A4 were replaced by new stainings in Figure 2C. CYP3A4 were positively stained in epithelial layers, comparing with no stained in submucosal cells’ cytoplasmic, of the XF-HIOs. In the revised comments, we presented the additional stainings with two different antibodies for CYP3A4 in XF-HIOs and the Sigma’s antibody, which were used in Figure 2C of the revised manuscript, for CYP3A4 in human intestine. We also added antibodies’ negative controls (isotype control IgG antibodies-mouse; D2-40 and CYP3A4 and rabbit;SLC10A2, SLC15A1, ABCB1, CES1, CES2 and Ezrin ) in new Supplementary figure 5.

3. The authors state several times in their manuscript that their organoids are outward oriented, but the images provided do not definitively show this. Also, no epithelial polarity or junction markers are shown to demonstrate orientation.

Action/Response
We are grateful for the reviewer’s comments. We may have short explanations on the epithelial layers facing outward. Figure 1b, 1d and 2c show that the epithelium layers of XF-HIOs face outwards. We also added Ezrin staining as Supplementary Figure 1c. Ezrin positively localized to an apical part of the epithelial layers in the XF-HIOs.

4. The CYP3A4 activity luciferase approach used in this study appears to have been based on the assumption that the organoids have a functional barrier. Organoid permeability data should be included. In addition, the CYP3A4 activity data is not very convincing as shown. One important control would be data from XF organoids not exposed to the proluciferin substrate. In addition, because the authors are proposing that this culture system would be suitable for drug assessment, it would be important to show that the CYP3A4 activity could be modulated (up- and/or down-regulated) in response to a treatment condition.

Action/Response
We are grateful for the reviewer’s critical comments. We repeated CYP3A4 activity luciferase analysis for XF-HIOs additionally using CYP3A4 inhibitor, Ketoconazole. The CYP3A4 activity of XF-HIOs was markedly suppressed by Ketoconazole. We replaced by this data in Figure 4b in the revised manuscript.

Our thanks to the reviewers for their valuable comments, queries and suggestions. These have all helped us to improve our manuscript. We look forward to hearing from you regarding your decision. We would be glad to respond to any further questions or comments that you may have.

**AFTER THE REVISIONS THE ASSOCIATE EDITOR DECIDED TO ACCEPT.**