EMBO reports

Mouse fetal intestinal organoids: new model to study epithelial maturation from suckling to weaning

Marit Navis, Tânia Martins Garcia, Ingrid B. Renes, Jacqueline L.M. Vermeulen, Sander Meisner, Manon E Wildenberg, Gijs R. van den Brink, Ruurd M. van Elburg and Vanesa Muncan

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Editor: Esther Schnapp

Transaction Report:

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

1st Editorial Decision 10 May 2018

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee comments that is pasted below.

As you will see, the referees acknowledge that the study is potentially interesting. However, they also point out that it should be developed a little further, and that the data need to be strengthened. They suggest several experiments to do so. In order to save some time, I am making a decision now asking you to address all referee concerns. We normally first ask the referees for cross-comments in order to identify the most crucial concerns that should be addressed. If you cannot address all concerns, I can still go back to the referees and ask them for cross-comments.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Supplementary figures, tables and movies can be provided as Expanded View (EV) files, and we can

offer a maximum of 5 EV figures per manuscript. EV figures are embedded in the main manuscript text and expand when clicked in the html version. Additional supplementary figures will need to be included in an Appendix file. Tables can either be provided as regular tables, as EV tables or as Datasets. Please see our guide to authors for more information.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution. In order to avoid delays later in the process, please read our figure guidelines before preparing your manuscript figures at: http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In this manuscript Muncan and colleagues characterize the evolution of organoids derived from fetal small intestine. They show that overtime these fetal organoids autonomously undergo changes similar to those observed during the suckling-weaning transition in vivo. I found the study interesting yet descriptive and somewhat preliminary. Although I have no major criticisms regarding the experimental part, the study would gain priority if authors could include functional analyses and further extend the current observations. I suggest several lines of improvement:

1. Does ablation of Blimp-1 alter the maturation of fetal organoids as suggested by author's previous studies?

2. How does dexamethasone influence the suckling-weaning transition at the transcriptional level? Authors have the opportunity to perform chromatin analysis (e.g. Chip) of glucocorticoid receptor in fetal organoids and analyze the mechanism by which it controls the differentiation program.

3. Does the ISC dependency on Wnt signaling evolve during the suckling-weaning transition? How is the Lgr5+ cell compartment modulated?

4. Authors did not include a morphological characterization of the fetal organoid culture overtime (beyond a few bright field pictures). I suggest that authors perform detailed analyses of the cell types and organoid organization, assess changes in structure and composition over time and compared these observations to the in vivo situation. Electron microscopy analyses could be useful in this regard.

5. Authors must investigate further the formation of budding structures. Do they coincide with the appearance of Paneth cells? Are both organoid types composed of Lgr5+ cell and differentiated cells?

Referee #2:

Navis and colleagues employ the recently established in vitro technique to grow mouse intestinal epithelial organoids from isolated stem cells to study the transit period between suckling and weaning. Their work analyses the expression levels of selected mostly metabolic marker genes in addition to enzyme activities and immunostaining in in vitro grown fetal-derived and adult-derived organoids. They (i) demonstrate the strong influence of age- on epithelial gene expression by global transcriptomics, (ii) confirm age-dependent expression of metablic marker genes by RT-PCR and enzymatic activity analysis, (iii) illustrate the time-dependent maturation of fetal-derived organoids phenocopying mature, adult epithelium after 30 days (again by RT PCR and enzyme activity assays), (iv) demonstrate the maturation promoting activity of cortisol and the anatomical differences (but not marker differences) between early and late culture of fetal-derived organoids. The work uses a state of the art technique to unravel an important question and illustrates the epithelium intrinsic maturation program. Given some minor/

Major comments:

1. In figure 1, stem cell organoids of fetal versus adult origin are compared with total intestinal tissue of neonate and adult mice. The transcriptional profile of primary isolated intestinal epithelial cells of neonate versus adult has previously been published and is freely accessible (GEO GSE35596). Could the presented data be extended to include primary epithelial cells in addition to total intestinal tissue? This might significantly improve the overlap e.g. in Fig. 1C.
2. Page 6, 3rd paragraph: Lct expression follows a kinetic that differs very significantly from Ass1 and Blimp1. Pleased modify text and specify.
3. Please consider to include the expression level of untreated adult organoids in Fig. 4 for better illustration.
4. Figure 5C: are all organoids similarly positive/negative? Please provide a quantitative analysis of the number of organoids that stain pos/neg. if not.
5. If the authors reuse data from previous figures, please mention in the figure legend. For example, the data of control adult organoids in Supplemental Fig. 4c seems to be the same values as Fig. 3i-l. Please indicate throughout the manuscript.
Minor comments:
1. Page 6, 2nd paragraph: Defcr is a synonym of Defa (alpha defensin). No receptor for alpha defensins exists to the best knowledge of the reviewer.
2. The first paragraph in the introduction section says: `The most apparent structural changes...two weeks...In the following week, major functional changes...`. This means that the suckling-to-weaning transition has to be after P14. However, in the following text,it says: `around postnatal day 14 in mice (P14), the adaptation...`. Please be precise with the time points indicated.
3. Please add a reference to the sentence saying: `conditional deletion of Blimp-1 from mouse...` (page 3, 4th paragraph).
4. IIn the 2nd paragraph of page 6 of the result section, it says `In contrast to the neonatal..., and

trehalase (Treh) (Supplementary Fig. 2b and 2e)...`. However, Supplementary Fig. 2b is the histology images of Arginase 2. Please double check the figure and correct if necessary.
5. Please use the same style (i.e. either `Supplementary Fig. 2f-h` or `Supplementary Fig. 2f-2h`) to refer to the figures.
6. In the third part of the results section, it says `Similarly, FcRn...CRAMP (Fig. 2c and 2d) follow...`. However, CRAMP is shown neither in Fig. 2c nor 2d.
7. In paragraph 2, page 9, it says `In a sharp contrast, adult markers Sis and Treh...(Fig. 5d and 5e)`. However, Fig. 5e shows the marker Arg2.
8. In the figure legend of Fig. 5,
- you mention the time kinetic `day 3, 13, 20 and 28`. However, another kinetic is indicated in the figure itself: `day 3, 13, 20 and 30`. Please correct.
- Please include a scale bar in Fig. 5a, 5c, 5d and 5e.
- `representative microscopic images showing embryonic marker` is indicated in the legend. However, the marker is not included in the figure. Please correct.
9. In figure legend for the Supplementary Fig. 3, `in vitro` should be italic.
10. Please add scale bar in Supplementary Fig. 5e-g.
11. Page 7, 2nd paragraph typo: (At One)

Referee #3:

In this manuscript, the authors showed that organoid culture system is suitable for recapitulating intestinal maturation during suckling-to-weaning transition. More specifically, the authors focused on the intrinsic factors (Ass1, Blimp1, FcRn, Lct, Sis, Treh, Arg2), which are mainly associated with metabolic changes. The authors found that expression pattern (from qPCR) of the selected genes in D30 fetal organoid is similar to that of adult tissue (P42). Also, over time the expression of the genes in D30 fetal organoid becomes more alike to adult organoids. Therefore, the authors concluded that using the selected markers, one can study suckling-to-weaning transition using organoid.

Some concerns
1. From the gene expression profile data, it seems that D30 fetal organoid is more similar to D3 fetal organoid than to adult tissue. The authors are convinced that this confers maturation after prolong culture time, however it seems that the global gene expression in D30 organoid remains fetal-organoid-like. It would have been useful to include adult organoid gene expression array to see the comparison and to assess more carefully whether maturation of fetal organoid does occur.
2. It is not new that gene expression of fetal organoids changes in culture. In a recent gut paper, Kraiczy et al. already showed that methylation and gene expression undergo dynamic changes. The data the authors have shown here are not convincing enough that we have learnt something new besides the correlation of a few selected genes.
3. Authors did not consider the fact that different developmental features of different segments of the gut. In the Fordham et al. (2013) Cell stem cell paper, they showed that adult stem cells from the proximal intestine (Postnatal Day 3) mostly developed cystic organoids/ spheroids, whereas mid and distal intestine-derived ones developed mostly into budding organoids. It would therefore be interesting for the authors to test and compare over different time points the gene expression of Ass1, Blimp1, FcRn, Lct, Sis, Treh, Arg2 after establishing organoids from the different segments. qPCR of the different segments, rather than taking RNA from the whole tissue.
4. Immunohistochemistry of Ass1, Sis and Arg2 in fetal-organoids shown in Figure5 seems that the localization of the proteins is not so specific, except for Sis. The staining from tissues (Supp fig 1, 2) showed that the staining is quite specific to the villi/brush border. The authors should look in more detail the localization, whether it is similar in organoid and tissue.

1st Revision - authors' response 28 August 2018

Referee #1:

In this manuscript Muncan and colleagues characterize the evolution of organoids derived from fetal small intestine. They show that overtime these fetal organoids autonomously undergo changes

similar to those observed during the suckling-weaning transition in vivo. I found the study interesting yet descriptive and somewhat preliminary. Although I have no major criticisms regarding the experimental part, the study would gain priority if authors could include functional analyses and further extend the current observations. I suggest several lines of improvement:

1. Does ablation of Blimp-1 alter the maturation of fetal organoids as suggested by author's previous studies?
Transcription factor Blimp1 is expressed in intestinal epithelium during neonatal phase of life. In the current work we have utilized Blimp1 as a specific *in vivo* neonatal marker. We have investigated changes in Blimp1 expression during *in vitro* propagation of fetal epithelial organoids showing that it follows the *in vivo* expression pattern. Our manuscript illuminates many different aspects of *in vitro* epithelial maturation and articulates the main message that fetal organoids can be used to study/model intestinal epithelial maturation. In our opinion, culturing and examining the maturation pattern of intestinal organoids derived from Blimp1KO animals will not add much to the main message of our article. Reason for this is a complete absence of neonatal epithelial phenotype in Blimp1KO mice. Consequentially, Blimp1KO intestines are marked with the adult (mature) features and will generate adult organoids. We agree that the mechanism on how Blimp1 and other neonatal expressed genes contribute to the neonatal epithelial phase of intestinal development is worth investigating, but exceeds the scope of the current work where all of these are being used as neonatal markers.

2. How does dexamethasone influence the suckling-weaning transition at the transcriptional level? Authors have the opportunity to perform chromatin analysis (e.g. Chip) of glucocorticoid receptor in fetal organoids and analyze the mechanism by which it controls the differentiation program.
In our experiments, we have used dexamethasone as an example of an extrinsic factor that is shown and best studied in literature to accelerate maturation *in vivo*. Dexamethasone is a synthetic glucocorticoid and it exerts its effect via genomic and non-genomic routes. Indeed, we show that effects of dexamethasone *in vitro* were similar to these observed *in vivo*. For example, on the level of genomic regulation, we show a precocious increase of sucrase-isomaltase RNA levels after dexamethasone treatment. On a non-genomic level, we observed alterations in the activity of digestive enzymes like trehalase or arginase. Both are in accordance with the described specific effect of dexamethasone on sucrase gene activation and non-genomic protective effect on intestinal brush border enzymes (PMID: 6765934). Taking this into consideration performing Chip analyses of GR receptor in organoids will not fully reveal the effect of dexamethasone on precocious maturation. However, the fact that extrinsic factors such as glucocorticoids modulate certain aspects and timing of the suckling-to-weaning transition *in vitro* similarly to that described *in vivo*, further establishes the fetal organoid model for the investigation of such factors.

3. Does the ISC dependency on Wnt signaling evolve during the suckling-weaning transition? How is the Lgr5+ cell compartment modulated?
We have now investigated the expression pattern of Lgr5 during the course of the culture. In the early cultures, Lgr5 is expressed throughout intestinal epithelium. Coinciding with the appearance of Paneth cells, Lgr5 positive cells confine to crypt area in later cultures (Appendix Figure S2).
4. Authors did not include a morphological characterization of the fetal organoid culture overtime (beyond a few bright field pictures). I suggest that authors perform detailed analyses of the cell types and organoid organization, assess changes in structure and composition over time and compared these observations to the in vivo situation. Electron microscopy analyses could be useful in this regard.
We thank the reviewer for this suggestion and have now included a more detailed analysis of intestinal cell types in our culture model. These analyses showed that with exception of Paneth cells, all intestinal cell types are present at the beginning and after prolonged culture of fetal organoids. The results are presented in Appendix Figures S2 and S3.

5. Authors must investigate further the formation of budding structures. Do they coincide with the appearance of Paneth cells? Are both organoid types composed of Lgr5+ cell and differentiated cells?
We have investigated the presence of budding structures within each passage during the course of our culture, as previous reports (PMID: 24139758, 24139799) stated that fetal

epithelial intestinal cells grow as spherical structures that do not spontaneously transit to organoids. However, these cultures were initiated from earlier developmental stages (E14-16) that are reported to be composed of identical cells throughout the gut tube with little to none specification to various gut segments (PMID: 26260278). Within our culture conditions starting from E19 we observe spontaneous transition of spheroids to organoids as has been reported for adult cultures (PMID:19329995). Our study reveals that the budding structures are not *per se* a sign of maturation. We clearly observe the expression of adult markers in spherical organoids at late culture and neonatal markers in budding organoids at early culture. Therefore, we feel that description of organoid budding would be the description of the organoid culture in general and that it does not have an implication in suckling-to-weaning transition.

Referee #2:

Navis and colleagues employ the recently established in vitro technique to grow mouse intestinal epithelial organoids from isolated stem cells to study the transit period between suckling and weaning. Their work analyses the expression levels of selected mostly metabolic marker genes in addition to enzyme activities and immunostaining in in vitro grown fetal-derived and adult-derived organoids. They (i) demonstrate the strong influence of age- on epithelial gene expression by global transcriptomics, (ii) confirm age-dependent expression of metabolic marker genes by RT-PCR and enzymatic activity analysis, (iii) illustrate the time-dependent maturation of fetal-derived organoids phenocopying mature, adult epithelium after 30 days (again by RT PCR and enzyme activity assays), (iv) demonstrate the maturation promoting activity of cortisol and the anatomical differences (but not marker differences) between early and late culture of fetal-derived organoids. The work uses a state of the art technique to unravel an important question and illustrates the epithelium intrinsic maturation program. Given some minor/
Major comments:
1. In figure 1, stem cell organoids of fetal versus adult origin are compared with total intestinal tissue of neonate and adult mice. The transcriptional profile of primary isolated intestinal epithelial cells of neonate versus adult has previously been published and is freely accessible (GEO GSE35596). Could the presented data be extended to include primary epithelial cells in addition to total intestinal tissue? This might significantly improve the overlap e.g. in Fig. 1C.
We have extracted the GEO GSE35596 data set and included the comparison with our fetal organoid data. Indeed the gene set enrichment analyses showed significant overlap between primary isolated intestinal neonatal cells at day 6 and fetal organoids, whereas intestinal epithelium at day 21 is enriched in fetal organoids cultured for 30 days. These data are now included in Figure EV1C and D. Additionally, we transcriptionally profiled adult organoids cultured for 3 and 30 days which in PCA analyses cluster with fetal organoids cultured for 30 days (Figure 1A and Figure EV1A-B).
2. Page 6, 3rd paragraph: Lct expression follows a kinetic that differs very significantly from Ass1 and Blimp1. Pleased modify text and specify.
We have provided more extensive explanation in the text acknowledging the differences in expression between Lct and Ass1/Blimp1.
3. Please consider to include the expression level of untreated adult organoids in Fig. 4 for better illustration.
We have now included the expression of untreated adult organoids in Fig 4.
4. Figure 5C: are all organoids similarly positive/negative? Please provide a quantitative analysis of the number of organoids that stain pos/neg. if not.
We have included the quantitative analyses of positive/negative organoids in Fig 5C.
5. If the authors reuse data from previous figures, please mention in the figure legend. For example, the data of control adult organoids in Supplemental Fig. 4c seems to be the same values as Fig. 3i-l. Please indicate throughout the manuscript.
We have now indicated this.

Minor comments:
1. Page 6, 2nd paragraph: Defcr is a synonym of Defa (alpha defensin). No receptor for alpha defensins exists to the best knowledge of the reviewer.
We have corrected this in the current version of the manuscript.

2. The first paragraph in the introduction section says: `The most apparent structural changes...two weeks...In the following week, major functional changes...`. This means that the suckling-to-weaning transition has to be after P14. However, in the following text,it says: `around postnatal day 14 in mice (P14), the adaptation...`. Please be precise with the time points indicated.
We have better explained our intention to state that suckling-to-weaning transition onsets at two weeks of age and gradually extends till 4 weeks postnatal, a time point in development where intestinal maturation associated with suckling-to-weaning transition is complete.

3. Please add a reference to the sentence saying: `conditional deletion of Blimp-1 from mouse...` (page 3, 4th paragraph).
We have now provided the requested references.

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We have corrected this.

5. Please use the same style (i.e. either `Supplementary Fig. 2f-h` or `Supplementary Fig. 2f-2h`) to refer to the figures.
We have adjusted and synchronized the nomenclature of Figure style.

6. In the third part of the results section, it says `Similarly, FcRn...CRAMP (Fig. 2c and 2d) follow...`. However, CRAMP is shown neither in Fig. 2c nor 2d.
We have added the figure reference for CRAMP.

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We have corrected this.

8. In the figure legend of Fig. 5,
- you mention the time kinetic `day 3, 13, 20 and 28`. However, another kinetic is indicated in the figure itself: `day 3, 13, 20 and 30`. Please correct. **We have synchronized the time kinetics between the figure and the text.**
- Please include a scale bar in Fig. 5a, 5c, 5d and 5e. **We have included the scale bar**.
- `representative microscopic images showing embryonic marker` is indicated in the legend. However, the marker is not included in the figure. Please correct. **We have corrected this.**
9. In figure legend for the Supplementary Fig. 3, `in vitro` should be italic. **We have corrected this**.
10. Please add scale bar in Supplementary Fig. 5e-g. **We have included the scale bar.**
11. Page 7, 2nd paragraph typo: (At One). **We have corrected this.**

Referee #3:

In this manuscript, the authors showed that organoid culture system is suitable for recapitulating intestinal maturation during suckling-to-weaning transition. More specifically, the authors focused on the intrinsic factors (Ass1, Blimp1, FcRn, Lct, Sis, Treh, Arg2), which are mainly associated with metabolic changes. The authors found that expression pattern (from qPCR) of the selected genes in D30 fetal organoid is similar to that of adult tissue (P42). Also, over time the expression of the genes in D30 fetal organoid becomes more alike to adult organoids. Therefore, the authors concluded that using the selected markers, one can study suckling-to-weaning transition using organoid.

Some concerns
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We have now included the analyses of expression profiles of adult organoids cultured for 3 and 30 days (Figure 1A). These analyses further indicate that fetal intestinal organoids

cultured for 30 days show overall similarity to adult organoids at both time points of culture. Moreover, there is no difference between fetal organoids cultured for 30 days and adult organoids according to Pearson statistical correlation test. The additional data are represented in the modified Figure 1A and Figure EV1A and B.

2. It is not new that gene expression of fetal organoids changes in culture. In a recent gut paper, Kraiczy et al. already showed that methylation and gene expression undergo dynamic changes. The data the authors have shown here are not convincing enough that we have learnt something new besides the correlation of a few selected genes.
The novelty of our report is a careful description and analysis of *in vitro* maturation mimicking the suckling-to-weaning transition. Moreover, crucial intestinal functions associated with suckling-to-weaning transition are recapitulated *in vitro* at the same pace as *in vivo*. A few selected genes examined in detail in our study have a profound impact on intestinal brush border function and digestion of food. Additionally, we present evidence that, to some extent, suckling-to-weaning transition can be modulated *in vitro* by dexamethasone. This drug has a broad potential. We can now use this *in vitro* system for easier (compared to *in vivo*) investigations of factors that influence the dynamic transitioning of neonatal epithelial functions to adult. This is of direct importance for early life as well as later life health. Intestinal epithelial functions associated with the change of feeding must mature in a proper time frame to assure lifelong fitness of an organism (PMID:21878906). Deviation in the intestinal epithelial maturation processes might predispose to disease later in life.
Kraiczy *et al.*, have not studied/reported intestinal maturation of specific enzymatic functions associated with suckling-to-weaning transition neither reported the transitioning of neonatal to adult epithelium *in vitro*. Furthermore, the time frame in which *in vitro* maturation occurs has not been evaluated. Of note, a recent paper (PMID:29930978) reported that early fetal cultures do not transition to late fetal cultures and that both retain a stabile gene expression pattern (of selected markers) up to 20 passages. More careful comparative studies regarding human development are needed to describe the *in vitro* maturation of human tissues. This is of particular importance as in mice the early fetal derived intestinal organoids (E14-16) also remain stable in culture (PMID: 24139758, 24139799). Regarding the mouse comparative studies, the additional novelty we have reported here, is that late fetal intestinal cells, just prior to birth are capable of undergoing *in vitro* maturation process.

3. Authors did not consider the fact that different developmental features of different segments of the gut. In the Fordham et al. (2013) Cell stem cell paper, they showed that adult stem cells from the proximal intestine (Postnatal Day 3) mostly developed cystic organoids/ spheroids, whereas mid and distal intestine-derived ones developed mostly into budding organoids. It would therefore be interesting for the authors to test and compare over different time points the gene expression of Ass1, Blimp1, FcRn, Lct, Sis, Treh, Arg2 after establishing organoids from the different segments. qPCR of the different segments, rather than taking RNA from the whole tissue.
We have included the suggested analyses of proximal and distal tissue segments and found no difference in maturation pattern. Although the relative expression of some of the maturation genes differed between proximal and distal intestine, the overall maturation with respect to the increase/decrease of expression of specific markers occurred in a similar pattern and time frame. The data are presented in Appendix Figure S1.

4. Immunohistochemistry of Ass1, Sis and Arg2 in fetal-organoids shown in Figure5 seems that the localization of the proteins is not so specific, except for Sis. The staining from tissues (Supp fig 1, 2) showed that the staining is quite specific to the villi/brush border. The authors should look in more detail the localization, whether it is similar in organoid and tissue.
We have included better images as well as the quantification of the stainings. Of note, staining for argininosuccinate synthetase and arginase 2 is known to be cytoplasmic, in contrast to sucrase-isomaltase that is a brush border enzyme.

2nd Editorial Decision 22 October 2018

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees as well as referee cross-comments.

As you will see, while referee 1 feels that an analysis of organoids derived from Blimp1 KO mice should be added to the study, referees 2 and 3 agree that this is not necessary. We can therefore in principle accept your manuscript.

As discussed, please clarify what exactly the data shown in the figures are based on and on what exactly the statistics are based. If n<3 no error bars and p-values can be calculated, but single data points along with their mean can be shown instead. Data from representative experiments can be shown, if the experiment was repeated several times with similar results.

Fig 4E & F are called out before Fig 4C & D, please check that all figure panels are called out in the correct order.

Fig 1E - the text of the heatmaps is overlapping on the left side.

The Appendix tables are missing the 'S' in their nomenclature, and should be Appendix Tables S1 + S2, instead of S2 + S3. The Table EV1 is not part of the Appendix. Appendix Figs S1 and S4 are missing the statistical information in the legends.

I look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have any questions or comments.

REFEREE REPORTS

Referee #1:

Authors have failed to address several of my criticisms. It is particularly difficult to understand why they did not include the analysis of organoids derived from Blimp1 KO mice. This will reinforce the notion that organoids can be used to study postnatal development and the subsequent suckling to weaning transition.
I think that the manuscript contains very interesting information and that deserves to be published but it needs to be further developed.

Referee #2:

The authors have added new experimental data and significantly improved the mansucript. The questions raised have been adequately addressed. The manuscript in the present form is of great general interest and suitable for publication in EMBO Reports.

Referee #3:

The authors addressed comments and concerns from the reviewers really well. However, since the authors mentioned in the reply that early mouse fetal-derived intestinal organoids have been shown to remain stable in culture, it is therefore crucial to better define the starting material throughout the manuscript. One suggestion is to state the exact embryonic day, ie 'E19-derived fetal organoids...' to avoid confusion with early fetal-derived organoids.

Overall, the manuscript was very much improved. However, one should keep in mind that the type of manipulation is still limited for studying suckling-to-weaning transition. This is because one cannot control the initiation of suckling-to-weaning process in vitro. For future studies, it would be important to improve the manipulation strategy from drug/chemical treatments to more flexible and scalable methods such as genetic engineering [(conditional) knockout/over-expression models].

Cross-comments from referee 2:

I understand the point with the Blimp1 KO organoids and of course that would be interesting to look

at. However, the field is quite competitive and my feeling was that the insight provided is solid and of general interest and sufficient to warrant publication.

Cross-comments from referee 3:

I feel that the manuscript is ready to be accepted. I should have written my comments more clearly - sorry. Regarding the concern from the reviewer #1, I feel that further studies on the point may add some value but the overall novelty and value of the manuscript won't change much. Considering timing etc, I would rather recommend accepting the manuscript with additional discussion and few more modifications in the text.

2nd Revision - authors' response 2 November 2018

The authors performed all minor editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Vanesa Muncan
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2018-46221V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Effect size could not be pre-specified. There was no explicit power used. Experiments were repeated for ≥2 times with similar results, indicated in all figure legends. Organoids were derived from app. 15 fetuses per experiment. Microarray analysis was performed with four independent biological replicates.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	When treating organoids with dexamethasone, organoids were randomly divided within one culture to receive either control medium or medium with dexamethasone.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The investigators were not blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical test are indicated in figure legends and materials and methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. One-way analysis of variance was used to test if an observed change over time was significant compared to day 3 of culture, with a Tukey post-test. To compare differences between two different conditions, two-way analysis of variance was performed with a Bonferroni post-test. These tests do not assume normal distribution of the data.
Is there an estimate of variation within each group of data?	For all values, mean and standard error are given.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Information about manufacturers, catalog number and dilution used for all antibodies is provided in the materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Primary cell culture, described in methods section. All cultures in the lab are checked bi-monthly for mycoplasma infection.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Female pregnant 6 weeks old C57Bl/6J mice were obtained from Charles River.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal procedures complied with the guidelines of the EU and were approved by the Animal Welfare Body (ALC102556).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliant to the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A data availability section for the microarray results of this study is included in the materials and methods.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All applicable data displayed in expanded view and appendix.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA