Self-organizing *in vitro* mouse neural tube organoids mimic embryonic development

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AUTHORS: JiSoo Park and Matthias Lutolf

Dear Dr. Lutolf,

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. The referees have provided constructive suggestions that should be straightforward to address. Including additional quantitation and evidence of reproducibility will strengthen the study. Similarly, the study would benefit from data that characterise the spatial patterning of the organoids. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

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in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so

Reviewer 1

Advance Summary and Potential Significance to Field

In Park et al., the authors describe a protocol to produce elongating organoids that resemble multiple aspects of embryonic neural tube development. The authors use 3D culture of mouse embryonic stem cells with the addition of neural inducing medium to transition through epiblast to neural progenitor fates, observing changes in organoid morphology as embryonic fate progresses. They describe the morphological features highlighting similarities to the developing neural tube, as well as provide single cell RNA sequencing analysis and subsequent in situ hybridization to confirm the emergence of spinal cord neural progenitors using Hox gene expression. Finally, they describe maturation of both neuronal populations and neural crest populations migrating from the organoids at later timepoints. Overall, this paper offers an additional model for the in vitro study of the neural tube, contributing to the toolbox through which scientists can model spinal cord development. Additionally, the manuscript shows the emergence of a neural crest population, which has yet to be shown in a neural tube specific organoid.

Comments for the author

The advance of in vitro techniques to facilitate study of complex organ systems is highly valuable; however I would recommend that the authors provide quantification and additional characterization of their system to ensure its reproducibility and that the claims made of mimicking the neural tube are indeed valid.

1. Quantification: Of essential importance for a tools and techniques manuscript, the paper desperately needs n-values, significance statistics, and general quantification for the claims made. This is to ensure that the quality and reproducibility of organoid differentiations are recorded in a way that will allow other labs across the field to make use of the technique.

ACTIONABLE ITEMS -
a. How many organoids are in a biological replicate? The quantities of organoids are not explained in the manuscript other than in Fig4 for the hox gene in situs.
b. The images provided in Figure 2 are largely descriptive and would benefit from quantification. In particular, when the authors make claims on the polarity of aggregates, location of Edu incorporation, etc. Perhaps include measurement of distance from edge of organoid?
c. Where only the elongation index 4 (ie the most elongated phenotype) characterized or do all organoids polarize?
d. The studies in Figure 5 are descriptive and would benefit from quantification i.e. What is the ratio of neuronal maturing organoids to neural crest maturing organoids? Does it relate to their size? Elongation ratio? Density at which they are plated? How often does maturation not yield either mature neurons or neural crest? What is the A-P identity of the subsequent neurons and neural crest? What is the dorsal ventral identity of the matured neurons?

2. Characterization: Besides having a singular tube, one of the hallmarks of the neural tube is axis patterning in both the anterior-posterior as well as the dorsal-ventral axes. The paper would benefit from characterization of the key elements of A-P and D-V patterning to further establish its novelty and match the claims of a self-organizing neural tube organoid.

ACTIONABLE ITEMS -
a. From the in situ hybridization of hox genes, it is unclear whether the entire aggregate is one A-P identity or whether there is a gradient. Quantification of the in situs would help with this as well as explanation in the text. Is the possible polarization of Hoxb9 are simply more differentiated cells separating from neural epithelium cells or is this a true neural tube A-P polarity? In particular, Pax6 is used as a marker of spinal cord in the RNA sequencing in figure 4 and the staining in Figure 2B

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displays pax6 throughout the organoids while figure 1D shows it in only a portion. What are the other cell types that are pax6 negative? Again, is each organoid only one A-P identity or a combination? Further characterization is needed to show A-P patterning within a single organoid. Or the claims in the text need to be ameliorated.
b. The single cell RNA sequencing picked up on other cell types such as mesoderm, where are these cells located within the organoids and in what proportion?
c. Similarly, the D-V axis is not flushed out and is integral to spinal cord development. Are these organoids only ventral cell types? Dorsal? A cross-section with relevant markers for D-V patterning (Olig2, Nkx2.2, Pax6, Pax7, Foxa2, etc) would be important to demonstrate neural tube patterning beyond elongating cysts of un-patterned neural progenitors. Can any D-V relevant genes be picked up in the sequencing data? Does the D-V patterning change in the matured day 8 organoids?

Additional minor comments:

1. Figure 1C needs significance indications for qPCR as does figure S1.
2. In Figure 5D, to confirm the matured neurons are neural crest derived and not differentiating from a contaminating population of neural progenitors, the authors could stain for peripherin.

Overall, I really enjoyed the manuscript and following revision, I think that the system will significantly contribute to the advancement of the field.

Reviewer 2

Advance Summary and Potential Significance to Field

The manuscript by Jisoo Park describes a novel in vitro model of neural tube development, using mESCs, Matrigel and hardly any patterrning factors. The model involves self-elongating organoids with formation of tube cavity, A/P patterning and neural crest delamination, and it documents a very interesting feature of neural crest phagocytosis. The model is novel in demonstrating both neural A/P patterning as well as 3D elongated tube morphology at the same time. It's possible that this combination of features makes the model more suitable for studying neural crest delamination from the roof plate. The study is well performed and scientifically sound, and deserves publication in my view, although a few points need to be addressed. Importantly, it needs to be demonstrated if this differentiation phenotype is specific to the mESC line applied in the given study, or if it can also be transferred to other cell lines.

Comments for the author

Issues that should be addressed in the manuscript:
- Title: Since most new organoid papers being published today are based on human cells, it would be relevant to specify in the title that this study is based on mouse, i.e. "Self-organizing in vitro mouse neural tube organoids mimic embryonic development"

- It is curious that the model presented obtains caudalisation into the spinal cord in the absence of any exogenous RA or WNT activation. This naturally opens the question of whether the cell line used in this particular study has an intrinsic propensity towards caudalisation and whether other mESC cell lines can yield similar results. To clarify this, the authors should perform their differentiation protocol on at least two other cell lines and present key characterisation data from these cell lines in the manuscript.

- Figure 4A and Fig. S2: It's unclear if these two plots are showing the same data in two different formats - please clarify this. Also, it's unclear how the annotations of the clusters has been done, and several annotations here seem misleading. In particular:

o The cluster annotated as midbrain/hindbrain appears to be mis-annotated as it shows a clear expression pattern of postmitotic neurons with high Stmn2, which is a very specific postmitotic marker. No patterns in the "midbrain/hindbrain" cluster show indications of midbrain or hindbrain fate. Therefore, this annotation should be changed.

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o The "Neurectoderm" cluster would be more correctly annotated as "caudal neurectoderm" or simply as "hindbrain", as it is devoid of Otx2, and expresses medium/high levels of Hoxb2. In essence, the "Neurectoderm" and "Hindbrain" clusters look completely identical and differ only by the intensity of certain genes. I would suggest that these two clusters are either merged, or labelled simply as "Hindbrain 1 " and "Hindbrain 2". It would be relevant to include Hoxa1 and Hoxb1 in the heatmap plot as markers of the anterior hindbrain to clarify further the identity of these clusters.
- In Fig. 3A, there are clusters annotated as Epiblast, Surface Ectoderm and Posterior Mesoderm, but these are absent from Fig. 4A. Please specify in the text if these clusters have been manually removed in the heatmap shown in Fig. 4A.
- Please provide full gene lists of cluster-specific DEGs as an xls file together with the manuscript upon resubmission - this list is necessary in order to properly review the correctness of the clustering.

- It's stated in the Materials and Methods that a Sox1-GFP/Bra-Cherry double reporter line was used for this study. However, there is no mentioning of this reporter cell line in the Results section, and multiple figures show stainings in the red and green channels which would be expected to be confounded by the fluorescent reporters. Several issues need to be clarified here: How was this reporter line generated and validated? What is the source mESC line used for this reporter line? Was this reporter line used for all experiments, or only for some (if so, which other lines were used)? How is it validated that the fluorescent stainings performed have not been confounded by the fluorescent reporters? If this reporter line has been used, it should be applied to validate if the organoids truly contain neuromesodermal progenitors at early stages of differentiation, since this would be expected if spinal cord identities are present in the organoids.

- In Materials and Methods, please specify which algorithm was used to integrate the datasets. Please also describe the sequencing depth (reads per cell) obtained in the scRNAseq experiments.
- Fig. S4: Can the neural crest phagocytosis time lapse also be provided as a mp4 movie file?

Reviewer 3

Advance Summary and Potential Significance to Field

In this study, Park and Lutolf establish a 3D neural tube-like organoid model from mouse embryonic stem cells (mESCs). The organoids are generated by plating mESCs in a droplet of Matrigel. Subsequently, the cells are treated with bFGF and Activin to induce an epiblast like state and then allowed to differentiate in N2B27 medium. The 3D aggregates initially formed a cyst that further elongated into a neural tube-like structure. They further analyzed the cell types present in the organoids by single cell RNA sequencing of pooled organoids at day3 and day6. Their data suggested the presence of different cell types in the organoids and the transition from an epiblast like (day3) to neural progenitor state (day 6) partially resembling the in vivo process. The formation of a lumen, apicobasal polarity and presence of neural crest cells are very interesting features that these organoids developed. In the future, it would be nice to explore if the model could also be established with human ESCs. Overall, this is a nice additional organoid model to study early events associated with neural tube development. Especially, the presence of neural crest cells and delamination opens up the opportunity to study these mechanisms using an in vitro model with spatiotemporal resolution. My main concerns that need to be strengthened/clarified in the manuscript are the reproducibility of the model, interpretation of the data and in vivo relevance.

Comments for the author

Major points
The authors state that 'Herein, we present a self-organizing neural tube organoid that is similar in morphology, cell type composition, and patterning to the mouse embryonic neural tube.' This statement is not supported from the data presented in the manuscript. For example, there is no evidence of dorsoventral patterning in the organoids. Also there are no data to support the presence of signaling centers and gradients along the dorsal - ventral and anterior - posterior axis which are key features of the developing neural tube.

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An important concern in the organoid field is reproducibility. The authors should include data to prove the reproducibility of their model. No precise details about the number of organoids analysed and statistical methods used have been reported. This information should be included in the figures and material and methods. For example in Figure 1D. How many organoids have been analysed by immunofluorescence and possess similar expression patterns?

How many different mouse ESC lines have the authors used to generate the organoids? I can only find an SBr mESC line . Why the authors decided to use the specific reporter cell line? Is the protocol reproducible with wild type mESC lines? In the methods they state that the number of cells for the SBr line is 1125 cells /10ul. Does the number need to be adjusted depending on the line used? Is the number of cells plated in 3D important for the formation of the lumen?

The authors state that they induce the generation of neural tube like organoids from single mESCs. In the protocol they dissociate the mESCs and plate them in a droplet of Matrigel, which is clearly not indicative of single cell organoid formation. To support such conclusion, they will have to perform clonal experiments. Otherwise, they should rephrase in the abstract and manuscript.

Can the authors exclude the possibility that single organoids are forming from multiple mESCs or that there is a fusion of two or more organoids over time? They plate 1125 cells in 10ul of Matrigel when they start the process of aggregation.

How many organoids are usually forming in a single droplet of Matrigel? How similar they are in size? The formation of a lumen structure in the middle of the organoid is very clear from the images and videos. Is this consistent among different organoids? Are all the organoids developing a lumen structure? In figure S1 they present only data regarding the elongation efficiency, which is around 50%. What happens to all other organoids? Are they maintained as spheroids? What is the anterior-posterior identity of the organoids that do not elongate? Do they have a more anterior identity?

Is activin required for the generation of the epiblast like cells in this model? Have the authors tried to use only bFGF in the absence of externally supplemented activin?

How long are the maturation experiments? The authors state that they performed long term maturation experiments but I can only find data from organoids until day 8.
What is the evidence for the presence of mature neurons in the organoids? The authors should present immunofluorescence data of mature neurons, e.g. motor neuron markers like Islet1/ChAT, to prove their point.

Can the authors identify anterior and posterior regions organized in the same organoid? For example they should check a midbrain marker and one of the posterior Hox genes together to show that there is an AP patterning. To prove that there is an AP patterning they should also present immunofluorescence data for midbrain, hindbrain and spinal cord specific genes in the same organoid. In Figure 4D, Hoxb1 seems to be expressed throughout the organoid, which suggests that the organoids have a hindbrain identity in the absence of more anterior fates. Another important concern is that the single cell data are generated from the pooling of 100 organoids. This means that it is very difficult to prove that all the different populations presented in Figure 3, 4 and FigureS2 are present in individual organoids.

From the data in figure 4, it seems that the most posterior Hox genes expressed in these organoids are Hoxb9 and Hoxc9 at the transcript level. Lumbar Hox genes are not expressed e.g Hoxc10. This needs to be clarified in the manuscript. Also, to prove that there are spinal cord neurons present the authors should check for the co-expression of Hoxc6 (brachial) or Hoxc9 (thoracic) protein with a neural marker like b-tubulin.

In the supplementary figure 3, there is a cluster of posterior mesodermal cells. Have the authors checked if the mesodermal cells are organized in their organoids by immunofluorescence analysis? What is the developmental origin of these mesodermal cells? Are neuromesodermal progenitors induced at any point of organoid formation/ elongation? If not, what drives the elongation process in these organoids?

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The summary statement should be toned down because clearly the organoids are not similar to the in vivo neural tube. The authors could rephrase to partially resembling some aspects of the in vivo neural tube.
Also, the title needs to be adjusted.

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

The advance of in vitro techniques to facilitate study of complex organ systems is highly valuable; however I would recommend that the authors provide quantification and additional characterization of their system to ensure its reproducibility and that the claims made of mimicking the neural tube are indeed valid.

1.Quantification: Of essential importance for a tools and techniques manuscript, the paper desperately needs n-values, significance statistics, and general quantification for the claims made. This is to ensure that the quality and reproducibility of organoid differentiations are recorded in a way that will allow other labs across the field to make use of the technique.

[Q1] a. How many organoids are in a biological replicate? The quantities of organoids are not explained in the manuscript other than in Fig4 for the hox gene in situs.

=> Thank you for your comment. We have now stated the number of organoids and biological replicates in the figure or figure legend.

[Q2] b. The images provided in Figure 2 are largely descriptive and would benefit from quantification. In particular, when the authors make claims on the polarity of aggregates, location of Edu incorporation, etc. Perhaps include measurement of distance from edge of organoid?

Thank you very much for your comment. To quantify the apical localization of mitotic cells in the organoid, we have now performed immunofluorescence against apical membrane marker (Prominin-1) to tell the apical domain and M-phase cells (Phospho-Histone H3, pH3) and S-phase cells detection by EdU incorporation on 22 organoids in 3 biological replicates. The result is shown in Figure S2A and indeed the M-phase pH3+ cells were localised at the apical domain in the organoid while S-phase EdU+ cells do not show such localization. Also, we have included the time-lapse images that captured the cell division at the apical domain in Figure S2B and a time-lapse video of interkinetic nuclear migration in the organoid in Movie 4.

[Q3] c. Where only the elongation index 4 (ie the most elongated phenotype) characterized or do all organoids polarize?

Thank you for your question. The quantification of elongation index was performed only on the elongated organoids as the non-elongated ones stay spherical.

[Q4] d. The studies in Figure 5 are descriptive and would benefit from quantification i.e. What is the ratio of neuronal maturing organoids to neural crest maturing organoids? Does it relate to their size? Elongation ratio? Density at which they are plated? How often does maturation not yield either mature neurons or neural crest? What is the A-P identity of the subsequent neurons and neural crest? What is the dorsal ventral identity of the matured neurons?

Thank you very much for raising these important points. To answer these questions, we have performed mainly two types of analyses. First, we have quantified the ratio of the emergence of neural crest and neurons every day from D7 to D10 (Fig. 3C). From this quantification, we could observe that the neural crest cells emerge earlier than the neurons already from D7 and both neural crest cells and neurons emerged in the majority of the organoids from D9 onwards.

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To understand the specific identities of the neural crest and neurons that emerge from the mature organoids, we have performed scRNAseq of mature organoids at different timepoints: D6, D7, D8, and D10 (Fig. 4). From this single-cell analysis, we identified the cells migrating out from the organoid body as cranial neural crest cells and we captured the transcriptomic event of delamination through epithelial-to-mesenchymal transition (EMT) (Fig. 4, Fig S8). The serial timepoints of scRNAseq revealed neural differentiation from dorsal neural progenitors to dorsal interneurons (Fig. 4, Fig. S9). Specifically, we could demonstrate the trajectory of neural differentiation from dorsal progenitors (dp) 1 to dorsal interneurons (dI) 1 and dp2/3 to dI2 and dI3 showing each specific marker of the cell type. This result is in line with the previous data (Meinhardt et al., 2014; Ranga et al., 2016) where the neural cysts have a default dorsal identity without any ventralizing factor. As our protocol does not include any ventralizing factors, a default dorsal fate is expected and our data indeed shows that the neurons are dorsal neurons.

Furthermore, to test if the neural tube organoids can be induced to have ventral cell types by being exposed to ventralizing factors (as in vivo), we exposed the neural tube organoid to smoothened agonist (SAG) between D3 and D5 and compared the gene expression levels of dorsal-ventral patterning markers by qPCR (Fig. S7). We find that the control organoid (without SAG treatment) shows a higher expression of dorsal markers (such as Pax3 and Pax7) than the ventralized organoid (treated with SAG from D3 to D5) which shows higher expression of ventral markers (Olig2 and Foxa2). The immunofluorescence against the ventral markers OLIG2 and FOXA2 shows that the neural tube organoids lack ventral cell types, but can be ventralized to have ventral cell types. These data demonstrate that the neural tube organoid responds to morphogens similar to the native neural tube.

2.Characterization: Besides having a singular tube, one of the hallmarks of the neural tube is axis patterning in both the anterior-posterior as well as the dorsal-ventral axes. The paper would benefit from characterization of the key elements of A-P and D-V patterning to further establish its novelty and match the claims of a self-organizing neural tube organoid.

[Q5] a. From the in situ hybridization of hox genes, it is unclear whether the entire aggregate is one A-P identity or whether there is a gradient. Quantification of the in situs would help with this as well as explanation in the text. Is the possible polarization of Hoxb9 are simply more differentiated cells separating from neural epithelium cells or is this a true neural tube A-P polarity? In particular, Pax6 is used as a marker of spinal cord in the RNA sequencing in figure 4 and the staining in Figure 2B displays pax6 throughout the organoids while figure 1D shows it in only a portion. What are the other cell types that are pax6 negative? Again, is each organoid only one A-P identity or a combination? Further characterization is needed to show A-P patterning within a single organoid. Or the claims in the text need to be ameliorated.

Thank you very much for these very insightful comments. To address these key questions, we engaged in a collaboration with the lab of Dr. Gioele La Manno at EPFL (an expert in single cell analysis and spatial omics) who helped us to visualize the AP identity marker genes in single organoids. Specifically, we have used two different pairs of genes: Otx2 (midbrain) - Gbx2 (hindbrain) and Hoxb1 (hindbrain) – Hoxb9 (spinal cord) for single-molecule fluorescence in situ hybridization (smFISH). We observed that the majority of the organoids showed spatial patterning of anterior and posterior genes in a single organoid.

[Q6] b. The single cell RNA sequencing picked up on other cell types such as mesoderm, where are these cells located within the organoids and in what proportion?

Thank you for raising this point. We think that our newly performed scRNAseq experiments of mature organoids answer this question. The top markers of this “posterior mesoderm” cluster, such as Twist1 and Foxc1, turned out to also be the markers for pre-EMT neural crest cluster markers in Fig. 4. We therefore corrected our annotation for D6 cell types from “Posterior mesoderm” to “Mesenchyme”. The pre-EMT neural crest cell population makes up about 0.56% of D6 organoids.

[Q7] c. Similarly, the D-V axis is not flushed out and is integral to spinal cord development. Are these organoids only ventral cell types? Dorsal? A cross-section with relevant markers for D-V patterning (Olig2, Nkx2.2, Pax6, Pax7, Foxa2, etc) would be important to demonstrate neural tube patterning beyond elongating cysts of un-patterned neural progenitors. Can any D-V relevant genes

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be picked up in the sequencing data? Does the D-V patterning change in the matured day 8 organoids?

To test if the neural tube organoids can be induced to have ventral cell types by being exposed to ventralizing factors (as in vivo), we exposed the neural tube organoid to smoothened agonist (SAG) between D3 and D5 and compared the gene expression levels of dorsal-ventral patterning markers by qPCR (Fig. S7). We find that the control organoid (without SAG treatment) shows a higher expression of dorsal markers (such as Pax3 and Pax7) than the ventralized organoid (treated with SAG from D3 to D5) which shows higher expression of ventral markers (Olig2 and Foxa2). The immunofluorescence against the ventral markers OLIG2 and FOXA2 shows that the neural tube organoids lack ventral cell types, but can be ventralized to have ventral cell types. These data demonstrate that the neural tube organoid responds to morphogens similar to the native neural tube.

Additional minor comments:

[Q8] 1.Figure 1C needs significance indications for qPCR as does figure S1.

We have added the significance test result of Fig. 1C graph as a supplementary information (Table 1) and added the according information on Fig. S1 as well.

[Q9] 2. In Figure 5D, to confirm the matured neurons are neural crest derived and not differentiating from a contaminating population of neural progenitors, the authors could stain for peripherin.

Thank you very much for the suggestion. We have now performed the suggested experiment. Immunofluorescence against peripherin on D30 organoid indeed showed the peripheral neuron population (Fig. S6) which shows the multipotency of neural crest cells.

Reviewer 2 Comments for the Author:

Issues that should be addressed in the manuscript:

[Q10] Title: Since most new organoid papers being published today are based on human cells, it would be relevant to specify in the title that this study is based on mouse, i.e. “Self-organizing in vitro mouse neural tube organoids mimic embryonic development”

=> Thank you very much for your suggestion. We fully agree and have added “mouse” in the title as suggested.

[Q11] It is curious that the model presented obtains caudalisation into the spinal cord in the absence of any exogenous RA or WNT activation. This naturally opens the question of whether the cell line used in this particular study has an intrinsic propensity towards caudalisation and whether other mESC cell lines can yield similar results. To clarify this, the authors should perform their differentiation protocol on at least two other cell lines and present key characterisation data from these cell lines in the manuscript.
Thank you very much for this insightful comment. To explore the transferability of the model to other cell lines, we have applied the same protocol to four additional cell lines, including the wildtype of the cell line we used in the manuscript (Fig. S2). We also have quantified the efficiency of elongation of each cell line and performed immunofluorescence against neuroepithelial marker SOX1 and apical domain marker Prominin-1 to show that it is an elongated neuroepithelial tissue with a single neural canal-like lumen with apicobasal polarity. Our data shows that our protocol is not limited to one specific ESC line but seems broadly applicable. Having said that, we would like to point out that each cell line appears to have different optimal cell density to be embedded in Matrigel on D0. We have reported this in Table 1.

[Q12] Figure 4A and Fig. S2: It’s unclear if these two plots are showing the same data in two different formats - please clarify this.

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Thank you for your comment. Fig. 4A shows the markers of AP identity cell types in heatmap format to deliver the key message of the figure and Fig. S2 was to show the key markers of all the cell type clusters in general in a heatmap format. Please note that these figures have substantially changed based on the new data on spatial patterning of AP identity markers and scRNAseq with another biological replicate on D6.

[Q13] Also, it's unclear how the annotations of the clusters has been done, and several annotations here seem misleading. In particular:

oThe cluster annotated as midbrain/hindbrain appears to be mis-annotated as it shows a clear expression pattern of postmitotic neurons with high Stmn2, which is a very specific postmitotic marker. No patterns in the "midbrain/hindbrain" cluster show indications of midbrain or hindbrain fate. Therefore, this annotation should be changed.

Thank you very much for your valuable feedback. Indeed, the differential marker of the cluster was Egr2 and Ppp1r1b which are the rhombomere 3/5 markers. In the new scRNAseq analysis with two biological replicates, we have annotated this cell cluster as hindbrain.

[Q14] The "Neurectoderm" cluster would be more correctly annotated as "caudal neurectoderm" or simply as "hindbrain", as it is devoid of Otx2, and expresses medium/high levels of Hoxb2. In essence, the "Neurectoderm" and "Hindbrain" clusters look completely identical and differ only by the intensity of certain genes. I would suggest that these two clusters are either merged, or labelled simply as "Hindbrain 1 " and "Hindbrain 2". It would be relevant to include Hoxa1 and Hoxb1 in the heatmap plot as markers of the anterior hindbrain to clarify further the identity of these clusters.

Thank you very much for assessing the data in detail. We fully agree have and have corrected this. We wrongly annotated the marker Crabp1 as neuroectoderm marker. A deeper analysis having the scRNAseq data of mouse embryo from Pijuan-Sala et al. (doi: 10.1038/s41586-019-0933-9) as the reference, Crabp1 expression was already biased to posterior cell type like hindbrain and spinal cord.

[Q15] In Fig. 3A, there are clusters annotated as Epiblast, Surface Ectoderm and Posterior Mesoderm, but these are absent from Fig. 4A. Please specify in the text if these clusters have been manually removed in the heatmap shown in Fig. 4A.

Thank you for comment. As we answered on Q12, the focus of Fig. 4 was the AP identity of the cells in the organoid, thus, only the related cell types were projected as a heatmap. Please note that the format of the figures has been largely changed according to the new data about spatial patterning of AP identity markers and scRNAseq with another biological replicate on D6.

[Q16] Please provide full gene lists of cluster-specific DEGs as an xls file together with the manuscript upon resubmission – this list is necessary in order to properly review the correctness of the clustering.
Thank you for your feedback. We have provided the markers of each cluster in table 7 and 8 in the supplementary data.
[Q17] It's stated in the Materials and Methods that a Sox1-GFP/Bra-Cherry double reporter line was used for this study. However, there is no mentioning of this reporter cell line in the Results section, and multiple figures show stainings in the red and green channels which would be expected to be confounded by the fluorescent reporters. Several issues need to be clarified here: How was this reporter line generated and validated? What is the source mESC line used for this reporter line? Was this reporter line used for all experiments, or only for some (if so, which other lines were used)? How is it validated that the fluorescent stainings performed have not been confounded by the fluorescent reporters? If this reporter line has been used, it should be applied to validate if the organoids truly contain neuromesodermal progenitors at early stages of differentiation, since this would be expected if spinal cord identities are present in the organoids.

Thank you very much for raising these questions. The reporter cell line was developed by Deluz et al. and all the related information is described in details in the referenced original paper:

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doi:10.1101/gad.289256.116 (Deluz, C. et al. A role for mitotic bookmarking of SOX2 in pluripotency and differentiation. Genes Dev. 30, 2538–2550 (2016)).

Initially, we hypothesized that the elongation of the organoid would have happened by the axial elongation of neuromesodermal progenitor (NMP) like in the tail bud of the embryo. For this reason, we started the experiments with this cell line and performed scRNAseq based on this cell line to identify the cell types. After all the analysis of scRNAseq and immunofluorescence, we found out that the organoid does not express Brachyury throughout the culture and we could never find cells that are positive for both SOX2 and Brachyury (Fig. S5). But as we performed the scRNAseq with this cell line, we continued all the experiments with this cell line to be consistent. And the colour of the immunofluorescent images does not necessarily match to the fluorophore colour that we used. The immunostaining was always done avoiding green channel except SOX1 antibody as we know we have SOX1 expression, else it was done in far-red channel or red channel as we know we don't have any Brachyury expression (Fig. S5A). Importantly (see our answer to Q11 above), the formation of neural tube organoids happens reproducibly in different mESC lines.

[Q18]-In Materials and Methods, please specify which algorithm was used to integrate the datasets. Please also describe the sequencing depth (reads per cell) obtained in the scRNAseq experiments.

Thank you for your feedback. We have added more detailed information about the scRNAseq in the Materials and Methods.

[Q19]-Fig. S4: Can the neural crest phagocytosis time lapse also be provided as a mp4 movie file? Sure, we have provided as Movie #6.

Reviewer 3 Comments for the Author:

Major points:

[Q20] The authors state that 'Herein, we present a self-organizing neural tube organoid that is similar in morphology, cell type composition, and patterning to the mouse embryonic neural tube.' This statement is not supported from the data presented in the manuscript. For example, there is no evidence of dorsoventral patterning in the organoids. Also there are no data to support the presence of signaling centers and gradients along the dorsal – ventral and anterior – posterior axis which are key features of the developing neural tube.

=> Thank you for your feedback. We agree and have modified this statement to hopefully better capture what we have achieved: 'We present here a self-organizing neural tube organoid that mimics the mouse embryonic neural tube in terms of its morphology, cell type composition, and aspects of patterning'.

[Q21] An important concern in the organoid field is reproducibility. The authors should include data to prove the reproducibility of their model. No precise details about the number of organoids analysed and statistical methods used have been reported. This information should be included in the figures and material and methods. For example in Figure 1D. How many organoids have been analysed by immunofluorescence and possess similar expression patterns?
Thank you very much for raising this important point. We have performed additional quantifications. We have quantified the efficiency of organoid formation in Fig. S1 and Fig. S2 to demonstrate the reproducibility across different cell lines. Also, the AP patterning of the organoid has been shown in Fig. 2C. Please see also our more detailed answers to Q1 and Q11.

[Q22] How many different mouse ESC lines have the authors used to generate the organoids? I can only find an SBr mESC line. Why the authors decided to use the specific reporter cell line? Is the protocol reproducible with wild type mESC lines?

We have now generated organoids with four additional cell lines, including the wild type mESC (Fig. S2). The reason for using Sox1-GFP/Brachyury-mCherry mESC line was to detect potential NMP cells in the organoid based on our hypothesis on the mechanism of axial elongation. We kept performing

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experiments with this cell line to be consistent. Please see also our more detailed answers to Q11 and Q17.

[Q23] In the methods they state that the number of cells for the SBr line is 1125 cells /10ul. Does the number need to be adjusted depending on the line used? Is the number of cells plated in 3D important for the formation of the lumen?

Thank you for the question. Indeed, the optimal cell density of each cell line varies and we find this parameter to be very important in neural tube organoid development. If the cell density is too high, the efficiency of elongation decreases. On the contrary, if the cell density is too low, the organoid loses apicobasal polarity and do not form a lumen.

[Q24] The authors state that they induce the generation of neural tube like organoids from single mESCs.

In the protocol they dissociate the mESCs and plate them in a droplet of Matrigel, which is clearly not indicative of single cell organoid formation. To support such conclusion, they will have to perform clonal experiments. Otherwise, they should rephrase in the abstract and manuscript. Can the authors exclude the possibility that single organoids are forming from multiple mESCs or that there is a fusion of two or more organoids over time? They plate 1125 cells in 10ul of Matrigel when they start the process of aggregation. : Thank you for bringing up this key question. To address it, we have performed time-lapse imaging experiments that in our view convincingly demonstrate that neural tube organoids emerge from single cells. Please see Movie 1 and Figure 1B. We would like to point out that similar single cell-derived neural cyst organoids have been described previously by the lab of Elly Tanaka and our own lab (see for example Meinhardt, A. et al. 3D Reconstitution of the Patterned Neural Tube from Embryonic Stem Cells. Stem Cell Rep. 3, 987–999 (2014) and Ranga, A. et al. Neural tube morphogenesis in synthetic 3D microenvironments. Proc. Natl. Acad. Sci. 113, E6831–E6839 (2016).

[Q25] How many organoids are usually forming in a single droplet of Matrigel? How similar they are in size?

Thank you for your question. The quantification of organoid formation efficiency and the size is shown in Fig. S1C,D.

[Q26] The formation of a lumen structure in the middle of the organoid is very clear from the images and videos. Is this consistent among different organoids? Are all the organoids developing a lumen structure?

Yes, lumen formation is highly consistent in our system. Culturing mESCs in Matrigel, a basement membrane-mimicking gel, promotes apicobasal polarity and lumen formation (see also Bedzhov and Zernika-Goetz, Cell, 2014; Meinhardt et al., Stem Cell Reports, 2014; Ranga et al., PNAS, 2016). The organoids express multiple apical domain markers (such as Prominin-1 and ZO-1) at the lumen and apically localized mitotic cells display interkinetic nuclear migration.

[Q27] In figure S1 they present only data regarding the elongation efficiency, which is around 50%. What happens to all other organoids? Are they maintained as spheroids? What is the anterior-posterior identity of the organoids that do not elongate? Do they have a more anterior identity? Thank you for raising this point. Yes, the rest of the organoids retains a spherical cystic structure. In Fig. S6, we have explored the difference between elongated and non-elongated tissues by scRNAseq analysis. We think that the key difference is that the elongated organoids have a higher proportion of spinal cord cell types and also express much higher posterior Hox genes.

[Q28] Is activin required for the generation of the epiblast like cells in this model? Have the authors tried to use only bFGF in the absence of externally supplemented activin?

Thank you very much for this insightful question. Indeed, we could obtain self-elongating neural tube organoid with bFGF in the absence of Activin-A. However, the efficiency of elongation was much lower in bFGF only, as shown below:

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[Q29] How long are the maturation experiments? The authors state that they performed long term maturation experiments but I can only find data from organoids until day 8.

In this experiment setup, 'maturation' refers to organoid cultures that are longer than D6. Time-course images of organoid from D6 to D10 show that neural crest cells emerge first and then neurons emerge (Fig. 3C). For this reason, immunostaining against neural crest marker (AP2α, SOX10, NESTIN) was done on D8 and neuronal marker (BRN3a and TUJ1) on D10 organoid. To identify the specific cell types that emerge upon maturation, we performed scRNAseq on D6, D7, D8, and D10 (Fig. 4). We have also performed maturation experiments up to 30 days to explore the multipotency of neural crest cells that emerged from the organoids (Fig. S8).

[Q30] What is the evidence for the presence of mature neurons in the organoids? The authors should present immunofluorescence data of mature neurons, e.g. motor neuron markers like Islet1/ChAT, to prove their point.

Thank you very much for this important feedback. Please see our answer to Q4 above. Neural tube organoids without ventral morphogen stimulation have a default dorsal fate. scRNAseq of mature organoids show that the neurons are dorsal interneurons (dI1 expressing Lhx9, dI2 expressing Lhx1, and dI3 expressing Tlx3). scRNAseq data of mature organoids show Isl1+ and ChAT+ cells. However, the number of cells is very low (please see figure below). Nevertheless, Fig. S11 in our view clearly demonstrates neural differentiation along the trajectory from Sox2+ cells to Tubb3+ cells.

[Q31] Can the authors identify anterior and posterior regions organized in the same organoid? For example they should check a midbrain marker and one of the posterior Hox genes together to show that there is an AP patterning. To prove that there is an AP patterning they should also present immunofluorescence data for midbrain, hindbrain and spinal cord specific genes in the same organoid. In Figure 4D, Hoxb1 seems to be expressed throughout the organoid, which suggests that the organoids have a hindbrain identity in the absence of more anterior fates. Another important concern is that the single cell data are generated from the pooling of 100 organoids. This means that it is very difficult to prove that all the different populations presented in Figure 3, 4 and FigureS2 are present in individual organoids.

Thank you very much for this very insightful feedback that was also raised by reviewer 1 (Q5). To address this key question, we engaged in a collaboration with the lab of Dr. Gioele La Manno at EPFL (an expert in single cell analysis and spatial omics) who helped us to visualize the AP identity marker genes in single organoids. Specifically, we have used two different pairs of genes: Otx2 (midbrain) - Gbx2 (hindbrain) and Hoxb1 (hindbrain) – Hoxb9 (spinal cord) for single-molecule fluorescence in situ hybridization (smFISH). We observed that the majority of the organoids showed spatial patterning of anterior and posterior genes in a single organoid.

[Q32] From the data in figure 4, it seems that the most posterior Hox genes expressed in these organoids are Hoxb9 and Hoxc9 at the transcript level. Lumbar Hox genes are not expressed e.g Hoxc10. This needs to be clarified in the manuscript. Also, to prove that there are spinal cord neurons present the authors should check for the co-expression of Hoxc6 (brachial) or Hoxc9 (thoracic) protein with a neural marker like b-tubulin.
Thank you very much for your precious feedback on the spinal cord neurons. In the revised manuscript, we have now clarified that the Hox genes are expressed from Hox1 to Hox9 level. To detect Hoxc6 and Hoxc9, we have tried immunofluorescence using anti-HOXC6 antibody (sc-376330, Santa Cruz Biotechnology) and anti-HOXC9 antibody (5B5-2, DSHB), but we have not seen a clear signal. Instead, we could detect Hoxc6+ Tubb3+ cells and Hoxc9+ Tubb3+ cells from scRNAseq data of mature organoids (please see the figure below).

[Q33] In the supplementary figure 3, there is a cluster of posterior mesodermal cells. Have the authors checked if the mesodermal cells are organized in their organoids by immunofluorescence analysis? What is the developmental origin of these mesodermal cells? Are neuromesodermal progenitors induced at any point of organoid formation/ elongation? If not, what drives the elongation process in these organoids?

Thank you very much for raising these important questions that were also raised by the other reviewers (Q6 and Q17). We were also intrigued by the small mesoderm population in our initial

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scRNAseq dataset. We have now performed additional scRNAseq experiments of mature organoids and we think that our new data helped to provide additional insights. The top markers of this “Posterior mesoderm” cluster, such as Twist1 and Foxc1, turned out to be also the markers for pre-EMT neural crest cluster markers in Figure 4. From this, we have corrected our annotation for D6 cell types from “Posterior mesoderm” to “Mesenchyme” which implies the pre-EMT neural crest cell population in the organoid and the population is about 0.56% of D6 organoids.

Initially, we speculated that the elongation of the organoids could be driven via axial elongation of neuromesodermal progenitors (NMPs), like in the tail bud of embryo. However, after all the analysis of scRNAseq and immunofluorescence, we found out that the organoid does not express Brachyury throughout the culture and we never found cells that are double positive cells for SOX2 and Brachyury (see Fig. S5). We concluded that the axial elongation in our system is not driven by NMPs. Looking at the spontaneous cell rearrangements of the neuroepithelial cells of the organoids (see Movie 1), we assume that elongation involves a convergent extension process. As convergent extension is regulated by the planar cell polarity pathway, we have compared the expression level of key genes in D6 elongated and non-elongated (NE) structures. This analysis showed that the planar cell polarity genes are much highly expressed in elongated (please find the figure below). More in-depth studies are needed to elucidate the mechanism of this intriguing morphogenesis of the organoids as we think it does not elongate the same way as other axial elongating in vitro model such as gastruloids.

[Q34] The summary statement should be toned down because clearly the organoids are not similar to the in vivo neural tube. The authors could rephrase to partially resembling some aspects of the in vivo neural tube. Also, the title needs to be adjusted.

We have toned down the summary statement as follows: “Self-elongating neural tube organoids recapitulate key aspects of the morphology, anterior-posterior patterning, neural crest emergence and neural differentiation of mouse embryo in vivo by self-organization”. We hope that this better reflects what we have done. We respectfully disagree on the title and suggest to keep it as we think it is accurate. However, we have made the title more narrow by adding ‘mouse’ as we have not demonstrated human organoid derivation.

Resubmission

First decision letter

MS ID#: DEVELOP/2022/201052

MS TITLE: Self-organizing in vitro mouse neural tube organoids mimic embryonic development

AUTHORS: Matthias Lutolf, JiSoo Park, Irina Khven, Gioel La Manno, and Hao-An Hsiung

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. As you will see the referees request several clarifications but are otherwise satisfied that the revisions address the issues they initially raised. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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Reviewer 1

Advance summary and potential significance to field

The authors Park et al. describe an additional method by which to generate 3D neural tube organoids that mimic aspects of dorsal neural tube development including dorsal progenitor marker expression, the emergence of mature neurons and the emergence of neural crest cells. Overall, the organoid system is very well classified and relatively equivalent to previously reported systems for neural tube development with polarity, lumenization, AP patterning. Similar to previous systems there is an inability to self organize into the complete dorsal ventral patterning found in the neural tube without additional signalling added to their protocol. However, the emergence of a neural crest population from an in vitro organoid system is very interesting and currently unique to this system. Futhermore, the classification done in the article demonstrates reproducibility of neural crest emergence and maturation that shows the system is a valuable tool for the study of neural crest in vitro. As such, I would recommend for publication as a tools and techniques article.

Comments for the author

Minor comments:
-Please modify the language of the paper to ensure it is clear that your system as is can only recapitulate either the dorsal or the ventral cell types of the neural tube dependent on the protocol used.
-please extend the discussion to include why there may be absence on full D-V patterning in this system and how its development will strengthen the field.
-Does this system have evidence of the roof plate population? The authors show maturing neurons of and neural crest in the single cell RNA seq data, but it is never explicitly discussed whether a roof plate population also forms.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Park et al. has been revised and has addressed many of the reviewer comments. However, there are still a number of issues which were only addressed in the response letter to the reviewers and not in the actual manuscript.

Comments for the author

These last issues should be resolved before the manuscript is publication-ready:

- Regarding Q11, the authors state in the response letter that they have tested the protocol on 4 additional cell lines, however, Figure S2 only includes data from 3 additional lines – please clarify. The correct names of the mESC lines must be stated in Fig S2 as well as in the methods section. References to the original publications on the cell line derivation and/or information on the derivation sites and method must also be provided in the Methods section.
- Regarding Q12, the authors must specify in the figure legends when the same data set is analysed and presented in different ways. I.e. for supplemental figures using the same scRNAseq as already presented in primary figures, this must be specified in the legend.
- Regarding Q15, the authors have replied to the question in the response letter, but they have failed to address this lack of clarity in the manuscript.
For all figures presenting scRNAseq data, it must be clearly specified in the figure legend if the data shown has been pre-selected and if certain clusters have been removed. This applies in particular to Fig. 4, but may also apply to other figures.
- Regarding Q17, the authors have replied to the question in the response letter, but have failed to address the lack of clarity in the manuscript. Even though the cell line is published, the Methods section should include a brief summary of the Sox1/Bra reporter cell line, including whether it is a fusion or a reporter cell line (heterozygous/homozygous) and which mESC line it is based on.

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It should also be clarified that this cell line was used to generate data in all figures except for Fig. S2. In addition, the immunostaining section should describe how the authors have handled immunofluorescent channels during stainings given that the cells potentially can contain both GFP and mCherry.

- Tables 7 and 8 were provided upon request, but there's no reference or description of the content in these tables neither in the main manuscript nor in the Supplementary Information document. This must be fixed
- The discussion section should elaborate on the absence of CHIR and RA in the patterning protocol presented here compared to other published protocols on spinal cord organoids/gastruloids
- The discussion section should further speculate on the absence of NMPs in the organoids, since this conflicts with several other published papers describing the generation of spinal cord fates.

Reviewer 3

Advance summary and potential significance to field

The authors describe here a 3D self-organizing neural tube-like model developed from mouse embryonic stem cells. The neural tube organoids generate over time neural crest cells and neurons. This tractable in vitro model opens up new opportunities to study early morphogenetic events and early cell fate specification of neural tube-like cells. In the future, the model could be also used to study early neurodevelopmental diseases and diseases that affect the migration of neural crest cells.

Comments for the author

The authors have addressed my concerns and I am happy to support the publication of the revised version of the manuscript in Development.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

The authors Park et al. describe an additional method by which to generate 3D neural tube organoids that mimic aspects of dorsal neural tube development including dorsal progenitor marker expression, the emergence of mature neurons and the emergence of neural crest cells. Overall, the organoid system is very well classified and relatively equivalent to previously reported systems for neural tube development with polarity, lumenization, AP patterning. Similar to previous systems there is an inability to self organize into the complete dorsal ventral patterning found in the neural tube without additional signalling added to their protocol. However, the emergence of a neural crest population from an in vitro organoid system is very interesting and currently unique to this system. Futhermore, the classification done in the article demonstrates reproducibility of neural crest emergence and maturation that shows the system is a valuable tool for the study of neural crest in vitro. As such, I would recommend for publication as a tools and techniques article.

Reviewer 1 Comments for the Author:
Minor comments:
-Please modify the language of the paper to ensure it is clear that your system as is can only recapitulate either the dorsal or the ventral cell types of the neural tube dependent on the protocol used.
-please extend the discussion to include why there may be absence on full D-V patterning in this system and how its development will strengthen the field.

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→ Thank you very much for the feedback. We have now modified the language and added an extended discussion to clarify these points.

-Does this system have evidence of the roof plate population? The authors show maturing neurons of and neural crest in the single cell RNA seq data, but it is never explicitly discussed whether a roof plate population also forms.

→ Thank you very much for pointing out about the roof plate population. We have checked the roof plate markers of mouse neural tube on our scRNAseq data based on Delile et al. (Development 2019, doi:10.1242/dev.173807). Roof plate-specific markers such as *Lmx1a*, *Msx1*, *Msx2*, and *Wnt1* were distinctively expressed in the pre-EMT neural crest cell clusters, both cranial and vagal/trunk neural crest cell. This indeed indicates that the neural crest cells emerge from the roof plate-fated cells in the organoid, as *in vivo*. We have added this in the revised version with a new supplementary figure S12.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript by Park et al. has been revised and has addressed many of the reviewer comments. However, there are still a number of issues which were only addressed in the response letter to the reviewers and not in the actual manuscript.

Reviewer 2 Comments for the Author:
These last issues should be resolved before the manuscript is publication-ready:

-Regarding Q11, the authors state in the response letter that they have tested the protocol on 4 additional cell lines, however, Figure S2 only includes data from 3 additional lines - please clarify. The correct names of the mESC lines must be stated in Fig S2 as well as in the methods section. References to the original publications on the cell line derivation and/or information on the derivation sites and method must also be provided in the Methods section.

→ Thank you for your comment. We have clarified all the correct names and the references of the cell lines in both Materials and Methods section and the supplementary figure S2.

-Regarding Q12, the authors must specify in the figure legends when the same data set is analysed and presented in different ways. I.e. for supplemental figures using the same scRNAseq as already presented in primary figures, this must be specified in the legend.

→ Thank you very much for the precise comment. We agree that adding descriptions specifying which data set analysis that each of the supplementary figure support will help a lot. We have specified on the figure legends of the supplementary figures Fig. S5, S10-13.

-Regarding Q15, the authors have replied to the question in the response letter, but they have failed to address this lack of clarity in the manuscript. For all figures presenting scRNAseq data, it must be clearly specified in the figure legend if the data shown has been pre-selected and if certain clusters have been removed. This applies in particular to Fig. 4, but may also apply to other figures.

→ Thank you for your concerns. However, we have neither pre-selected nor removed the cell clusters in the figures.

-Regarding Q17, the authors have replied to the question in the response letter, but have failed to address the lack of clarity in the manuscript. Even though the cell line is published, the Methods section should include a brief summary of the Sox1/Bra reporter cell line, including whether it is a fusion or a reporter cell line (heterozygous/homozygous) and which mESC line it is based on.
It should also be clarified that this cell line was used to generate data in all figures except for Fig. S2. In addition, the immunostaining section should describe how the authors have handled immunofluorescent channels during stainings, given that the cells potentially can contain both GFP and mCherry.

→ Thank you for your comments. We have added all the missing information in the revised manuscript.

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-Tables 7 and 8 were provided upon request, but there's no reference or description of the content in these tables neither in the main manuscript nor in the Supplementary Information document. This must be fixed

Thank you very much for the important comment. Indeed, the references for cell identification are missing. We have added the description about the references on the Materials and Methods section for single cell RNA sequencing data analysis and the supplementary information.

-The discussion section should elaborate on the absence of CHIR and RA in the patterning protocol presented here compared to other published protocols on spinal cord organoids/gastruloids
-The discussion section should further speculate on the absence of NMPs in the organoids, since this conflicts with several other published papers describing the generation of spinal cord fates.

→ Thank you very much for the important feedback. We have added the description of differences between our system and other organoids in a new and extended Discussion section.

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors describe here a 3D self-organizing neural tube-like model developed from mouse embryonic stem cells. The neural tube organoids generate over time neural crest cells and neurons. This tractable in vitro model opens up new opportunities to study early morphogenetic events and early cell fate specification of neural tube-like cells. In the future, the model could be also used to study early neurodevelopmental diseases and diseases that affect the migration of neural crest cells.

Reviewer 3 Comments for the Author:
The authors have addressed my concerns and I am happy to support the publication of the revised version of the manuscript in Development.

→ Thank you very much.

Second decision letter

MS ID#: DEVELOP/2022/201052

MS TITLE: Self-organizing in vitro mouse neural tube organoids mimic embryonic development

AUTHORS: Matthias Lutolf, JiSoo Park, Irina Khven, Gioel La Manno, and Hao-An Hsiung
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

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