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# Self-organized developmental patterning and differentiation in cerebral organoids

Magdalena Renner, Madeline A. Lancaster, Shan Bian, Heejin Choi, Taeyun Ku, Angela Peer, Kwanghun Chung and Juergen A. Knoblich

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**Review timeline:**

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| Submission date: | 04 May 2016 |
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## 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 03 June 2016

Thank you again for the submission of your manuscript entitled "Developmental patterning and differentiation in cerebral organoids" and for your patience during the review process. We have now received the reports from two referees, which I copy below.

As you can see from their comments, both referees are supportive of your work, but point out to a number of significant concerns that will require your attention before your manuscript can be published in The EMBO Journal. I will not repeat here the referee concerns, but in summary, both referees agree that the data presented would benefit from a better presentation and analysis, both from an statistical perspective and in terms of quality of the figures. I believe the concerns of the referees are reasonable and addressable, but please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points.

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

REFEREE REPORTS

Referee #1:

Since the pioneering work of Yoshiki Sasai and colleagues on the self-organisation of cerebral

 

cortex-like tissue in 3D ESC cultures and the first report of the generation of cerebral organoids by the Knoblich lab in 2013, in vitro models of human brain development have been adopted by many labs. A notorious difficulty with cerebral organoid cultures, however, is their variability. This study examines the spatial organisation and sequential generation of different cell types in organoids, focusing on forebrain-like tissues, and provides a mode detailed characterization of this aspect of organoid development than in the initial paper. It also examines the mechanisms underlying the spatial patterning of organoids by describing the presence of regions similar to the heme and the pallial-subpallial boundary (PSPB), two signaling centres that pattern the developing forebrain. Finally the paper provides some assessment of the heterogeneity of organoids, mostly in their spatial organisation. This study will be of interest to the many labs using this model. However, many aspects of the study are quite preliminary and a more thorough analysis is required before the paper can be published.

Major issues:

1) The identification of the PSPB/anti-heme in organoids should include an analysis of the expression of signaling molecules expressed in the PSPB in vivo (e.g. Sfrp2, Fgf7, Nrg1/3) to determine whether it has the patterning properties of the PSPB in vivo. Similarly, other signals than Wnt2b should be analysed in the heme (Wnts and BMPs) to demonstrate that it can act as a signaling centre as the heme in vivo. The results should be reported quantitatively, i.e. the numbers of hemes or PSPBs that express these signaling molecules should be indicated. It is currently unclear whether all hemes identified in organoids express Wnt2b. Negative results (absence of expression of a signaling molecule present in the PSPB or heme in the mouse forebrain) should be mentioned so that the readers gets a sense of where organoids recapitulate forebrain development, but also where they don't.

2) The authors argue that the presence of signaling centres in organoids influences the identity of adjacent regions. To support this idea, they should show a graph displaying the correlation between the presence of a hem or a PSPB and an adjacent cerebral cortex or GE.

3) The description of the temporal patterning of the organoids, i.e. the sequential generation of different neuronal subtypes, lacks an analysis of variability between experiments and between organoids within an experiment, similar to what is shown for spatial patterning in Fig. 2D. A graph should be included, preferably with information about the timing of generation of the different subtypes (i.e. whether Ctip2 and Satb2 neurons are generated at different times in different organoids).
Similarly, the variability of astrogenesis and oligodendrogenesis in organoids should be documented in a graph, and the generation of astrocytes and oligodendrocytes should be confirmed with at least a second marker (eg S100ß for astrocytes, Olig1 or NG2 for oligodendrocyte lineage cells). Also, is there a correlation between presence of a cerebral cortex or a GE and production of astrocytes or oligodendrocytes ?

4) The analysis of neuronal morphology is unconvincing. The pictures of Map2+ neurons are too small and should be magnified. If organoids can be electroporated, pictures of sparsely electroporated GFP+ neurons would be preferable as they would provide a better illustration of the complexity of neuronal morphologies. The text should mention that dendrites are not neatly aligned as they are in the cortex in vivo.

Minor points:

5) It should be mentioned somewhere (e.g. in the legend of Fig. 1) that Pax6 and Tbr2 are expressed in different, non-overlapping populations of cortical progenitors.

6) I presume the green signal along the cortical ventricular surface in Fig. 1C is background. This should be mentioned in the legend.

7) In Fig. 3D, the Lmx1a+ TTR- hem tissue is not in contact with Pax6+ ventricular progenitors. This raises the question of whether hem signals can diffuse and pattern the cortical tissue and should be discussed.

 

8) The legend of Fig. 2D mentions analysis of organoids between 30 and 70 days of culture. Could different types of organoids in Fig. 2D (radial, dorsal, etc) represent different stages of organoid development? The influence of age of organoids on spatial patterning should be discussed.

9) Gaspard et al., 2008 is not an appropriate reference for corticogenesis in vivo, page 8, last paragraph. A better reference would be Molyneaux et al. 2007 Nature Reviews Neuroscience.

10) Cajal Retzius cells are not generated by the cortical VZ but by specialized progenitors located at the edge of the cortex, including in the cortical hem and the PSPB. The characterisation of the expression of Reelin (p. 8, last paragraph) should therefore be moved to the previous section on spatial patterning of organoids. The first neurons produced by cortical progenitors are preplate neurons, which are eventually located superficially in layer one and deeply in the subplate. Unfortunately, there is no unambiguous marker for preplate or subplate neurons.

11) It is clear from Figure 4 that deep layer and upper layer neurons are generated sequentially in organoids, but also that they are not organised in well segregated layers as in the cortex in vivo. This should be mentioned in the text.

12) The references to the glia limitans and glial scar at the end of the Results section are too far fetched. GFAP+ astrocytes have not been analysed thoroughly enough to support such comparisons.

13) The discussion on temporal patterning in organoids could include a comparison between the timing of generation of deep layer and superficial layer neurons in organoids and in 2D cultures and other types of 3D cultures.

Referee #2:

In this manuscript Renner et al. present an extension and more detailed analysis of their initial description of the cerebral organoids. This systems holds the promise to be extremely powerful for the analysis of human brain development and also the aetiology of human developmental neurological disorders. One of the major challenges of the system is the extensive variability between experiments and individual organoids and whether each cerebral organoid contains both ventral and dorsal structures. The authors use immunostaining and 3D reconstruction to demonstrate that some organoids contain both dorsal and ventral cortical structures. They go on to show that signalling centres can be established in these organoids that potentially determine medial, dorsal, lateral and ventral forebrain tissue in the cultures. This they show is likely due to the formation of a complex and continuous ventricular-like system through the tissue. In general this is an interesting and necessary advance of the original description. In order that cerebral organoids can be used for quantitative analysis of gene function or phenotype, it is critical to either make the system more homogeneous or develop techniques and markers that can be used to evaluate changes. However, there are some issues that need to be addressed.

Main comments
1. Figure 1: the live imaging of the choroid plexus is not very convincing and the mages in 1L are not useful as it is not clear on the printed figures and only with imagination on the screen, what the arrows are pointing to.
2. Figure 1: What proportion of organoids show dorsal, ventral and putative hippocampal structures?
3. The authors should discuss the variability between experiments. Where, in their opinion does this variability originate.
4. The authors should show, or at least discuss what the radial non-drosal and the no-radial tissue organoids are or will generate.
5. Results page 6 Figure 2B-C: Foxg1 staining is not shown.
6. Results page 8 paragraph 2. It is not clear how many organoids were analysed. The authors should make clear the total number of organoids analysed and the percentages that are positive for particular criteria.
7. 4 out of the 19 organoids showed hem and PSPB. What is the percentage of total organoids analysed? It would be good to give the exact number of organoids that do not have hem or PSPB, have hem but no PSPB, have PSPB but no hem and those that have both. If dorsal/ventral patterning

 

happens, does it require/induce hem and PSPB or neither?
8. The neuron morphology data are not convincing in their current form. The authors need to quantify the radial morphology calculating the average angle to the organoid surface for example.
9. The organoids obviously do not form a layer cortical plate as the Satb2 and Ctip2+ neurons are intermingled in Figure 4, the authors need to discuss this.
10. Figure 5: the images are not sufficiently high quality and the O4 staining is not convincing. Why did they not use other astrocytic markers such as GLAST and S100-beta in panels A and B.

Minor comments.

The manuscript is not well edited. One example is the citations in the text. The abbreviation PSBP was defined twice. Some sentences: for example Discussion page 11 paragraph 3 last sentence...Further large-scale sequencing efforts like (Kerwin et al. 2010).... Need to be modified.

Figure 3C is cited out of sequence.

Figure 5 panels are labelled with lower case letter.

1st Revision - authors' response 01 December 2016

**Detailed response to reviewers' comments**

We would like to thank the reviewers for their constructive comments on our manuscript. In response to their concerns, we included additional experiments and modified the text. Below, we provide a detailed point-by-point response to all their comments.

The major new experiments are:

1. We performed a more thorough analysis of the organizing centers, in particular the cortical hem. We did stainings to quantify the expression of the signaling molecule Bmp6 in hem structures found within cerebral organoids. We observed, that all hem structures that were also stained for BMP6, were also positive for LMX1a on serial sections. Using antibody staining instead of in situ hybridization allowed analysis of tissues that were processed for immunohistochemistry and that had been previously characterized by forebrain identity staining. Furthermore, we improved our data presentation in general and included more quantifications (hippocampus, Nkx2.1 ventral forebrain).

2. We performed a more detailed analysis of the sequential generation of neurons and glia in organoids of 30 to 160 days of age and did more detailed quantifications.

3. We performed electroporation of a membrane-GFP to sparsely label neurons and visualize their morphology. This allowed us to confirm previous results obtained by antibody staining and further characterize neuronal morphology.

**Referee #1:**

Since the pioneering work of Yoshiki Sasai and colleagues on the self-organisation of cerebral cortex-like tissue in 3D ESC cultures and the first report of the generation of cerebral organoids by the Knoblich lab in 2013, in vitro models of human brain development have been adopted by many labs. A notorious difficulty with cerebral organoid cultures, however, is their variability. This study examines the spatial organisation and sequential generation of different cell types in organoids, focusing on forebrain-like tissues, and provides a mode detailed characterization of this aspect of organoid development than in the initial paper. It also examines the mechanisms underlying the spatial patterning of organoids by describing the presence of regions similar to the heme and the pallial-subpallial boundary (PSPB), two signaling centres that pattern the developing forebrain. Finally the paper provides some assessment of the heterogeneity of organoids, mostly in their spatial organisation. This study will be of interest to the many labs using this model. However, many aspects of the study are quite preliminary and a more thorough analysis is required before the paper can be published.

 

1) The identification of the PSPB/anti-heme in organoids should include an analysis of the expression of signaling molecules expressed in the PSPB in vivo (e.g. Sfrp2, Fgf7, Nrg1/3) to determine whether it has the patterning properties of the PSPB in vivo. Similarly, other signals than Wnt2b should be analysed in the heme (Wnts and BMPs) to demonstrate that it can act as a signaling centre as the heme in vivo. The results should be reported quantitatively, i.e. the numbers of hemes or PSPBs that express these signaling molecules should be indicated. It is currently unclear whether all hemes identified in organoids express Wnt2b. Negative results (absence of expression of a signaling molecule present in the PSPB or heme in the mouse forebrain) should be mentioned so that the readers gets a sense of where organoids recapitulate forebrain development, but also where they don't.

*Response: We appreciate the Reviewer's valuable suggestion, and we have now performed additional staining for signaling molecules to better characterize these putative signaling centers. Specifically, we tested a published antibody to Sfrp2 on samples on which we had previously identified PSPB, but unfortunately we were unable to detect specific signal. This could be due to an antibody problem, as we similarly failed to see positive signal on mouse brain sections, and we were unable to test the antibody on human brain slices. However, it is important to note that a signaling role for the PSPB is still to be definitively proven and the juxtaposition of dorsal and ventral forebrain is the defining characteristic, which we have shown.*

*For the hem we now performed staining for BMP6, as requested, and further include quantifications. Importantly, we found expression of BMP6 always in the same regions as LMX1a. Finally, we modified the discussion to address the potential roles of organizing centers further, but also to address challenges in identifying and characterizing them in organoids.*

2) The authors argue that the presence of signaling centres in organoids influences the identity of adjacent regions. To support this idea, they should show a graph displaying the correlation between the presence of a hem or a PSPB and an adjacent cerebral cortex or GE.

*Response: In order to identify organizing centers in our organoid samples we used both marker staining and structural cues of the tissue, namely the presence of choroid plexus and dorsal forebrain tissue curtailing the LMX1a/BMP6 positive. Since this was our definition of hem, all identified hem tissue displayed adjacent cerebral cortex. Similarly, our definition of PSPB was juxtaposition of dorsal and ventral tissue; therefore, all identified PSPB contained adjacent dorsal forebrain and ventral GE. We attempted to make that information more clear in the discussion.*

3) The description of the temporal patterning of the organoids, i.e. the sequential generation of different neuronal subtypes, lacks an analysis of variability between experiments and between organoids within an experiment, similar to what is shown for spatial patterning in Fig. 2D. A graph should be included, preferably with information about the timing of generation of the different subtypes (i.e. whether Ctip2 and Satb2 neurons are generated at different times in different organoids).
Similarly, the variability of astrogenesis and oligodendrogenesis in organoids should be documented in a graph, and the generation of astrocytes and oligodendrocytes should be confirmed with at least a second marker (eg S100ß for astrocytes, Olig1 or NG2 for oligodendrocyte lineage cells). Also, is there a correlation between presence of a cerebral cortex or a GE and production of astrocytes or oligodendrocytes ?

*Response: This is a very important point and we are happy to now include quantification of neuronal subtypes at various stages (included in Figure 4). This quantification was done on organoids from 15 independent experiments and shows the mean of at least 4 organoids from at least 2 experiments per time-point. The results nicely show the sequential generation of neuron subtypes. Furthermore, we also quantified the generation of astrocytes in organoids over time from the same 15 independent experiments which as expected revealed a delayed generation of astrocytes. We also include other markers for glial populations including GLT1 at an earlier time point for astrocytes, and Olig1.*

*Correlating the presence of dorsal forebrain or ganglionic eminence structures with the presence of astrocytes and oligodendrocytes at later stages of development is challenging, due to the fact that*

 

*radially organized germinal zones that could be used to infer identity are lost after around day 80. However, we quantified the number of organoids showing at least some astrocytes over time and found that GFAP+ cells with astrocyte morphology could be found in all organoids from around day 140 onwards.*

4) The analysis of neuronal morphology is unconvincing. The pictures of Map2+ neurons are too small and should be magnified. If organoids can be electroporated, pictures of sparsely electroporated GFP+ neurons would be preferable as they would provide a better illustration of the complexity of neuronal morphologies. The text should mention that dendrites are not neatly aligned as they are in the cortex in vivo.

*Response: As suggested by the reviewer, we have now performed electroporation for sparse labeling of neurons to analyze neuronal morphology and this is included in Figure 5.*

Minor points:

5) It should be mentioned somewhere (e.g. in the legend of Fig. 1) that Pax6 and Tbr2 are expressed in different, non-overlapping populations of cortical progenitors.
*Response: As suggested, this is now mentioned in the text and figure legend of Fig.1.*

6) I presume the green signal along the cortical ventricular surface in Fig. 1C is background. This should be mentioned in the legend.

*Response: Indeed, the ventricular staining is background in the absence of real signal. The figure legend has been modified to reflect this.*

7) In Fig. 3D, the Lmx1a+ TTR- hem tissue is not in contact with Pax6+ ventricular progenitors. This raises the question of whether hem signals can diffuse and pattern the cortical tissue and should be discussed.

*Response: We discuss in the main text that analysis of a complex 3D tissue by 2D sections can lead to difficulties in interpreting hem structures. We included in the discussion the possibility of signal diffusion through organoids, especially in the context of a complex 3D network of tissue connections.*

8) The legend of Fig. 2D mentions analysis of organoids between 30 and 70 days of culture. Could different types of organoids in Fig. 2D (radial, dorsal, etc) represent different stages of organoid development? The influence of age of organoids on spatial patterning should be discussed.

*Response: We included information on organoid age and the ability to form dorsal forebrain in the figure legend and methods.*

9) Gaspard et al., 2008 is not an appropriate reference for corticogenesis in vivo, page 8, last paragraph. A better reference would be Molyneaux et al. 2007 Nature Reviews Neuroscience.

*Response: We included the suggested reference.*

10) Cajal Retzius cells are not generated by the cortical VZ but by specialized progenitors located at the edge of the cortex, including in the cortical hem and the PSPB. The characterisation of the expression of Reelin (p. 8, last paragraph) should therefore be moved to the previous section on spatial patterning of organoids. The first neurons produced by cortical progenitors are preplate neurons, which are eventually located superficially in layer one and deeply in the subplate. Unfortunately, there is no unambiguous marker for preplate or subplate neurons.

*Response: We improved the description of Cajal Retzius cells to reflect this important point, however, since CR cells represent an early-born neuron type, still included them into the analysis with the other early and late born neuron types and glia.*

11) It is clear from Figure 4 that deep layer and upper layer neurons are generated sequentially in

 

organoids, but also that they are not organised in well segregated layers as in the cortex in vivo. This should be mentioned in the text.

*Response: We now include a description of this in the discussion with its implications.*

12) The references to the glia limitans and glial scar at the end of the Results section are too far fetched. GFAP+ astrocytes have not been analysed thoroughly enough to support such comparisons.

*Response: As suggested, we have removed these aspects of the text.*

13) The discussion on temporal patterning in organoids could include a comparison between the timing of generation of deep layer and superficial layer neurons in organoids and in 2D cultures and other types of 3D cultures.

*Response: We now include this comparison in the discussion, as well as comparison with in vivo.*

**Referee #2:**

In this manuscript Renner et al. present an extension and more detailed analysis of their initial description of the cerebral organoids. This systems holds the promise to be extremely powerful for the analysis of human brain development and also the aetiology of human developmental neurological disorders. One of the major challenges of the system is the extensive variability between experiments and individual organoids and whether each cerebral organoid contains both ventral and dorsal structures. The authors use immunostaining and 3D reconstruction to demonstrate that some organoids contain both dorsal and ventral cortical structures. They go on to show that signalling centres can be established in these organoids that potentially determine medial, dorsal, lateral and ventral forebrain tissue in the cultures. This they show is likely due to the formation of a complex and continuous ventricular-like system through the tissue. In general this is an interesting and necessary advance
of the original description. In order that cerebral organoids can be used for quantitative analysis of gene function or phenotype, it is critical to either make the system more homogeneous or develop techniques and markers that can be used to evaluate changes. However, there are some issues that need to be addressed.

Main comments
1) Figure 1: the live imaging of the choroid plexus is not very convincing and the mages in 1L are not useful as it is not clear on the printed figures and only with imagination on the screen, what the arrows are pointing to.

*Response: We intended to show the moving particles within choroid plexus as an indication of tissue functionality. However, we agree that the images shown make it difficult to see the particles. Since the particles can be seen clearly in the movies, we decided to keep the movie as supplemental data and move the still images to the supplemental figure.*

2) Figure 1: What proportion of organoids show dorsal, ventral and putative hippocampal structures?

*Response: As suggested, we now include quantifications of hippocampal and ventral structures into the legend of figure 1.*

3) The authors should discuss the variability between experiments. Where, in their opinion does this variability originate.

*Response: We now discuss the variability in the text to greater detail.*

4) The authors should show, or at least discuss what the radial non-drosal and the no-radial tissue organoids are or will generate.

 

*Response: We have now added a statement to the discussion regarding the presence of other non-forebrain regions in organoids.*

5) Results page 6 Figure 2B-C: Foxg1 staining is not shown.

*Response: We now include the FOXG1 staining into the supplemental figure.*

6) Results page 8 paragraph 2. It is not clear how many organoids were analysed. The authors should make clear the total number of organoids analysed and the percentages that are positive for particular criteria

*Response: We now include more thorough quantification of hem and PSPB into the manuscript.*

7) 4 out of the 19 organoids showed hem and PSPB. What is the percentage of total organoids analysed? It would be good to give the exact number of organoids that do not have hem or PSPB, have hem but no PSPB, have PSPB but no hem and those that have both. If dorsal/ventral patterning happens, does it require/induce hem and PSPB or neither?

*Response: We have now improved the reporting of which sets of organoid was analyzed for PSPB (same set as in figure 2) and hem. Hem was analyzed in experiments where > 50% of all organoids contained large, radially organized dorsal forebrain structures. In our analysis in organoids without large dorsal forebrain the identification of hem is impossible because the identification of hem in organoid samples depends on structural cues of the tissue, namely the presence of LMX1a/BMP6 positive tissue in between choroid plexus and dorsal forebrain tissue. Therefore, by definition, dorsal forebrain is found next to all identified hem tissues. We analyzed more samples and included them into the quantifications shown in figure 3.*

8) The neuron morphology data are not convincing in their current form. The authors need to quantify the radial morphology calculating the average angle to the organoid surface for example.

*Response: As described above and in response to a similar comment by Reviewer 1, we now include sparse labeling by electroporation of a membrane targeted GFP which reveals neurons with typical morphology, namely the presence of the primary dendrite. This data is now included in Figure 5.*

9) The organoids obviously do not form a layer cortical plate as the Satb2 and Ctip2+ neurons are intermingled in Figure 4, the authors need to discuss this.

*Response: This is a similar point to one raised by Reviewer 1, and we address this point in the discussion.*

10) Figure 5: the images are not sufficiently high quality and the O4 staining is not convincing. Why did they not use other astrocytic markers such as GLAST and S100-beta in panels A and B.

*Response: We now included additional marker stainings, including GLT1 at an earlier time point, and Olig1.*

Minor comments
The manuscript is not well edited. One example is the citations in the text. The abbreviation PSBP was defined twice. Some sentences: for example Discussion page 11 paragraph 3 last sentence...Further large-scale sequencing efforts like (Kerwin et al. 2010).... Need to be modified.
Figure 3C is cited out of sequence.
Figure 5 panels are labelled with lower case letter.

*Response: We improved editing of the manuscript, citing and figure labeling as suggested.*

 

2nd Editorial Decision 02 January 2017

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by both referees, who find that all their main concerns have now been addressed. There are just a few minor issues to be dealt with before formal acceptance here. Congratulations on a nice study!

1. Both referees have few remaining comments that should be addressed. They should be straightforward enough to resolve.
2. Please update the references according to the EMBO Journal style, order them alphabetically and finalise incomplete references. Multiple references should be included in the same brackets.
3. Images: please include a scale bar in Figures S1F and S2A.
4. The panels in Figure 5B are rotated in a peculiar fashion. If there is no particular reason for this style of presentation, I would recommend showing unrotated images.
5. Figure S2 is not referred to in the text, please update the callouts.
6. Please rename movie files into Movie EV1 and Movie EV2 and update the callouts in the text. Each movie file should be zipped together with the corresponding legend and uploaded as a dataset.
7. Please change supplemental figures into Expanded View Figures and change accordingly the callouts in the text. Please see our author guidelines on details about the content, purpose and preparation of Expanded View material (http://emboj.embopress.org/authorguide#expandedview).
8. Please assemble primer sequences into Table 2.

Let me know if you have any further questions regarding this or any previous points. You can use the link below to upload the revised version.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to seeing the final version.

REFEREE REPORTS

Referee #1:

In this revised version, the authors have significantly improved their manuscript. The variability in generation of dorsal forebrain, heme and PSPB in organoids between and within experiments is now reported. The generation of lower and upper layer neurons and astrocytes is characterised in greater details with a nice quantitative analysis of the timing of production of these cells types, and the morphology of differentiated neurons is also better illustrated.

Clearly, much more work will be needed to fully characterised cerebral organoids and the extent to which they model human forebrain development. However, this study makes important points that will be of great interests to researchers in this field and it is now sufficiently thorough to be published.

A minor remaining issue, the bibliographic list has multiple incomplete references and typos (Camp 2015; Lancaster 2013; Mariani 2015;

Referee #2:

In their revised manuscript Renner et al. have addressed most of my concerns and responded to my comments. However, I am sorry to insist but the authors really need to define more clearly for each experiment, either in the text, figure or legend exactly how many organoids were analysed and how many displayed the characteristics they observed. They need to state, how many organoids were generated, how many were included/excluded based on their criteria, of the selected (and maybe total) organoids how many showed the characteristic analysed. In response to my initial question "Figure 1: What proportion of organoids show dorsal, ventral and putative hippocampal structures?". In the rebuttal letter they claim to have added the numbers to the legend of Figure 1, but they added only some information. The authors use complex exclusion criteria for selecting the organoids that they analyse and use in their quantifications. The cerebral organoid is a powerful system but the reader and future users of the technique really need to know how many organoids display which

 

phenotypes in order that they can judge robustness and how many organiods they need to seed in order to obtain a statistically relevant number of organoids for their analyses. I suggest the authors add the information to each figure/legend or submit a supplemental table with the information. The authors should refer to the number and percentages of organoids and not just the number and percentage of experiments where they made a particular observation as this is not totally informative about the robustness of the system.

The quality of the GLT1, O4 and Olig1 images is very low and should be improved.

2nd Revision - authors' response 02 February 2017

**Point-by-point response to editorial and reviewers' comments**

Editorial concerns:
1. Both referees have few remaining comments that should be addressed. They should be straightforward enough to resolve.
*Response: Our responses to the various remaining concerns of the Reviewers are below. Please note that we have addressed all remaining comments.*

2. Please update the references according to the EMBO Journal style, order them alphabetically and finalise incomplete references. Multiple references should be included in the same brackets.
*Response: References have been checked and are formatted according to EMBO J style.*

3. Images: please include a scale bar in Figures S1F and S2A.
*Response: Scale bars included as requested.*

4. The panels in Figure 5B are rotated in a peculiar fashion. If there is no particular reason for this style of presentation, I would recommend showing unrotated images.
*Response: The rotation reflects the 3D view used for Imaris tracing. This is now clarified in the figure legend, and axes are shown in the figure.*

5. Figure S2 is not referred to in the text, please update the callouts.
*Response: This has been corrected.*

6. Please rename movie files into Movie EV1 and Movie EV2 and update the callouts in the text. Each movie file should be zipped together with the corresponding legend and uploaded as a dataset.
*Response: These changes have been incorporated as requested.*

7. Please change supplemental figures into Expanded View Figures and change accordingly the callouts in the text. Please see our author guidelines on details about the content, purpose and preparation of Expanded View material (http://emboj.embopress.org/authorguide#expandedview).
*Response: The supplemental data has been changed as requested.*

8. Please assemble primer sequences into Table 2.
*Response: Table 2 contains primer sequences as requested.*

Finally, thank you for providing a synopsis summary and image. I think that for the synopsis image it would be good to use something slightly more scientific that summarises the scientific information of the article. Alternatively, you can also use one of the images from the figures or a part thereof. Please take a look at http://emboj.embopress.org/ for some examples in previously published articles.
*Response: We have assembled a new synopsis image taking into consideration the editor's suggestions.*

Reviewer comments:
We would like to thank the reviewers for their constructive comments and careful reviewing of our manuscript. In response to their concerns, we updated incomplete references and created two expanded view tables summarizing all the quantifications done for the stainings performed for this manuscript.

 

Below, we provide a detailed point-by-point response to all their comments.

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Clearly, much more work will be needed to fully characterised cerebral organoids and the extent to which they model human forebrain development. However, this study makes important points that will be of great interests to researchers in this field and it is now sufficiently thorough to be published.

A minor remaining issue, the bibliographic list has multiple incomplete references and typos (Camp 2015; Lancaster 2013; Mariani 2015;

*Response: We completed and corrected the references.*

Referee #2:

In their revised manuscript Renner et al. have addressed most of my concerns and responded to my comments.

However, I am sorry to insist but the authors really need to define more clearly for each experiment, either in the text, figure or legend exactly how many organoids were analysed and how many displayed the characteristics they observed. They need to state, how many organoids were generated, how many were included/excluded based on their criteria, of the selected (and maybe total) organoids how many showed the characteristic analysed. In response to my initial question "Figure 1: What proportion of organoids show dorsal, ventral and putative hippocampal structures?". In the rebuttal letter they claim to have added the numbers to the legend of Figure 1, but they added only some information. The authors use complex exclusion criteria for selecting the organoids that they analyse and use in their quantifications. The cerebral organoid is a powerful system but the reader and future users of the technique really need to know how many organoids display which phenotypes in order that they can judge robustness and how many organiods they need to seed in order to obtain a statistically relevant number of organoids for their analyses. I suggest the authors add the information to each figure/legend or submit a supplemental table with the information. The authors should refer to the number and percentages of organoids and not just the number and percentage of experiments where they made a particular observation as this is not totally informative about the robustness of the system.

*Response: We now generated two expanded view tables summarizing the quantification data from Figure 1 - 3 (Table EV1) and Figure 4 (Table EV2). We mention the number of organoids analyzed for every staining performed and the outcome of the experiments. Since we were working with a very large number of organoid samples, we were unable to stain every sample for every marker. In the table we mention the selection criteria for performing certain stainings, for example, we only stained organoids for NKX2.1 that we had previously found to be positive for GSX2 or DLX2.*

*We added one sample to the quantification of hem tissue in figure 3E and 3F that we had previously stained for BMP6, but that had not been included into the quantification.*

The quality of the GLT1, O4 and Olig1 images is very low and should be improved.

*Response: A new image of GLT1 staining is shown with higher magnification, as well as insets showing higher magnification of O4 and Olig1 stainings.*

 

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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|---|
| Corresponding Author Name: Juergen A. Knoblich |
| Journal Submitted to: EMBO Journal |
| Manuscript Number: EMBOJ-2016-94700 |

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

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

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments 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 χ2 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.

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

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

## 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? | Because the statistics shown are descriptive of the population rather than a comparison of treatment groups, there were no effect size or power calculations. Sample size was simply the greatest number of experiments/organoids available. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | N/A |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | Experiments in which more than 50% of organoids did not fulfill predetermined quality criteria as described in Lancaster & Knoblich, 2014, were discontinued and not analyzed. For analysis of tissue identity, combinatorial marker stainings were performed as described in the text. |
| 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. | Organoids analyzed at different timepoints were chosen randomly for analysis from the population of organoids within the culture dish. Because of the use of an automated slidescanner for image acquisition, data from every organoid section were acquired and analyzed. No different treatment conditions were compared. |
| For animal studies, include a statement about randomization even if no randomization was used. | N/A |
| 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. | There was no comparison of different treatment groups performed |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | There was no comparison of different treatment groups performed |
| 5. For every figure, are statistical tests justified as appropriate? | No statistical tests/comparisons were performed. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | N/A |
| Is there an estimate of variation within each group of data? | N/A |
| Is the variance similar between the groups that are being statistically compared? | There was no comparison of different treatment groups performed |

## 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/

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| 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). | A list of all antibodies used in the study can be found in "methods". Antibodies were used in combinations and only regions, staining positive for an expected marker-combination, were considered. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | hES H9 cells were purchased from WiCell and confirmed to mycoplasma-free on a regular basis. |

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

## D- Animal Models

| | |
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| 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. | N/A |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | N/A |
| 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. | N/A |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | No human subjects |
| 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. | N/A |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | N/A |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | N/A |
| 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. | N/A |
| 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. | N/A |

## F- Data Accessibility

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | N/A |
| 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). | N/A |
| 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). | N/A |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | N/A |
| 22. 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. | N/A |

## G- Dual use research of concern

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|---|---|
| 23. 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. | N/A |