Rapid and robust directed differentiation of mouse epiblast stem cells into definitive endoderm and forebrain organoids

Daniel Medina-Cano, Emily K. Corrigan, Rachel A. Glenn, Mohammed Tarek Islam, Yuan Lin, Juliet Kim, Hyunwoo Cho and Thomas Vierbuchen
DOI: 10.1242/dev.200561

Editor: James Wells

Review timeline
Original submission: 26 January 2022
Editorial decision: 21 February 2022
First revision received: 15 June 2022
Accepted: 4 July 2022

Original submission

First decision letter

MS ID#: DEVELOP/2022/200561

MS TITLE: Rapid and robust directed differentiation of mouse epiblast stem cells into definitive endoderm and forebrain organoids

AUTHORS: Daniel Medina-Cano, Emily K Corrigan, Rachel A Glenn, Mohammed Tarek Islam, Yuan Lin, Juliet Kim, Hyunwoo Cho, and Thomas Vierbuchen

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. 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 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 this study Medina-Cano and colleagues demonstrate efficient and reproducible differentiation of mouse primed pluripotent stem cells (EpiSCs) into early definitive endoderm and into forebrain organoids. They achieve this by adaptation of protocols developed for human primed PSCs that have some underlying developmental logic. This is of value because although widely ignored, the relatively poor directed differentiation performance of EpiSCs has been a puzzle in the field. Notably, EpiSCs are considered most related to anterior primitive streak and therefore would be expected to differentiate directly and with high efficiency into definitive endoderm in response to developmental signals. The demonstration that this is indeed the case is therefore welcome, as is the finding of consistency between mouse and human primed PSC lineage induction. Although the study does not provide new insights into developmental mechanisms the topic is suitable for the Techniques and Resources section of Development. However, some additional data are required to consolidate the generality and utility of the findings.

Comments for the author

Essential revisions:
The main limitation of the study is that the EpiSCs used are all derived by in vitro conversion of ES cells and cultured in one particular condition.
? It cannot be certain that embryo-derived EpiSCs will respond equivalently.
Therefore, the authors should source embryo-derived EpiSCs and confirm this point.
? Use of feeder layers to maintain the EpiSCs is limiting because they are an unnecessary and potentially confounding factor. It is well established that EpiSCs can be reliably propagated without feeders (or KSR) on fibronectin coated dishes using activin and FGF with or without Wnt inhibition (Guo et al., 2009; Osorno et al., 2012; Tsakiridis et al, 2014). It will be helpful for wider ? user-friendly? adoption of their differentiation protocols that the authors show applicability to feeder-free EpiSCs.
? The authors must examine the responsiveness of EpiSCs cultured without Wnt/tankyrase inhibition. They imply throughout that this condition is a pre-requisite but present no evidence whether that is actually the case.
Production of functional definitive endoderm should be validated by further differentiation into e.g. pancreatic or hepatic lineages. The RNA-seq data on DE differentiation is a useful resource that could be exploited further, in particular for comparative analysis with embryo DE to corroborate identity. It would be helpful to see a global comparison in addition to the selected genes shown in Fig. S2E heatmap. It is also not clear how genes shown in the heatmap were selected - have they been cherry picked?
It is important to show that the forebrain organoid results are reproducible across EpiSCs of different genetic background, as shown for DE induction. Maybe the authors have done this but it is not apparent in the data presented.
The authors describe their EpiSCs as "primed ground state". I find this terminology unhelpful and potentially confusing to readers. The concept of primed PSCs is well understood in the community but there is neither justification nor criteria presented for use of "ground state" in the present context. "EpiSCs cultured in the presence of Wnt/Tankyrase inhibition" is clear and sufficient.
Additional suggestions:
In Figure 1 it would be helpful to include some comparative analysis with ES cells which should show that the EpiSCs are similar to one another and very different from ES cells.
Have the authors tested less than 16h for aPS induction? If not, they should either do so or else declare they have not done so.
Data in Fig 2B do not support the conclusion that a high concentration (100ng/ml) activin is required for maximal DE induction. At 24h with LDN there is little difference over the activin concentration range. This is important because 100ng/ml is not physiological.
The statement ?naïve PSCs can be converted into stable ground state EpiSC lines within 3-4 days? is not accurate. After 4 days cultures will be heterogeneous.
Moreover, without serial passaging it cannot be concluded that a stable stem cell population is established.
The statement that EpiSCs ?potentially have improved karyotypic stability compared to naïve PSCs? is unjustified without evidence (which is not in the cited paper about human PSCs).

ATAC-seq does not directly map cis-regulatory elements.
In vivo epiblast is developmentally dynamic from E4-E7.5. Therefore, the authors should declare the stage (E6.5) in the text relating to Fig 1F.
The start of the section on DE induction should state that the cells are first transferred to CDM.
What is meant by a ?wild-derived inbred strain??
It is unnecessary and appears rather self-serving to write that the Sasai protocols "were never widely adopted".
In the Introduction section on the requirement for naïve PSC to exit the naïve state to become responsive to inductive cues it would be appropriate to cite Mulas et al., SCR, 2017 which presents direct evidence for this. Similarly Tsakiridis et al., DEV, 2014 should be cited with regard to WNT pathway inhibition limiting spontaneous differentiation in EpiSC cultures. The subsequent reference to Najm et al. seems misplaced because that paper preceded the WNT pathway discovery.

Reviewer 2

Advance summary and potential significance to field

In this article, Medina-Cano et al. report on the derivation of 12 new EpiSC lines from mESCs. They characterize their stability and their similarity to previous lines and various embryo populations and confirm their similarity to epiblast. They also report on the variability between lines.
In a second step they take inspiration from protocols developed for human ESCs and use Wnt and activin and then activin alone to induce definitive endoderm.
They carefully optimize their protocol, systematically testing different doses of both pathway activators and the timing on one line. The analysis is quantitative, the conclusion is clear and further tested in two additional lines. Two rather important things remain unaddressed: (1) what type of endoderm is generated (2) would changes in the dosage and duration of the different growth factors and small molecules improve differentiation in the least efficient line. This would be interesting to know for future users but I don't view this as essential.
In the last part, the authors build on the original Eiraku/Sasai protocol to generate forebrain organoids from two of the lines. Based on a combination of markers, the identity of the cells produced is proposed to be forebrain including dorsal, caudo-medial telencephalon (hippocampus and cortical hem) and dorsal anterior diencephalon (prosomere 3 prethalamus). No comparison with endogenous cells is provided, which is a weakness of the manuscript.

In general the work is novel as very little has been done in general in mouse and even less starting from EpiSC. The work is well grounded on developmental principles, which makes the fit to the journal good. The investigation is solid and careful. The manuscript is well written and illustrated.
The most important weaknesses of the manuscript are (1) the feeling of a patchwork associating two different parts, one on endoderm in 2D, only 2 steps of development and one on brain in 3D with many developmental steps; (2) part 1 is conceptually not novel as the molecules and principles used are the same as in human.

Comments for the author

Page 1: "protocols for directed differentiation of mouse PSCs into defined lineages tend to be 29 slower" What do the authors mean. There have been less people working on such protocols indeed and therefore the field has been slower but as far as I know the protocols are not slower themselves. Yes, the beginning is slower because hES are closer to an epiblast state but then they tend to be slower at later stages. This is actually reported by the authors later in the paper. I don't think the statement is general enough to include in the abstract but the intro is correct.
Page 5: (Loh et al., 2014; Morgani and Hadjantonakis, 2020; Yiangou et al., 2018; Zorn and Wells, 2009). There are earlier publications for these two-stage protocols in human comprising a first step of Wnt/activin and then activin alone.
Fig 1DE. It is surprising that there are important transcriptional differences between lines derived from B6.129 and C57Bl/6J but little difference by ATAC seq. Do the authors have a tentative explanation?
Fig. 5C could be better exploited and linked to the rest of the figure by indicating markers on the map.
Note that some references are not complete.

It would be useful to elaborate in the discussion on the differences with the Eiraku protocol (others than the fact it starts from mESCs) that may explain why different brain areas are finally obtained. I could not find the movies, a pity! As a consequence I could not check them.

Reviewer 3

Advance summary and potential significance to field

Current protocols for the directed differentiation of mouse PSCs need optimization as they are slower and plagued by poor reproducibility compared to differentiation of hPSC. Unfortunately, so far, the broader developmental biology community has not invested much effort in improving these protocols, limiting our ability to characterize aspects of embryonic development that are not easily accessible and testable in vivo. Here Medina-Cano et al.; established and very thoroughly characterized two protocols for rapid and directed differentiation of mouse epiblast stem that can respectively generate 1) nearly pure definitive endoderm and 2) prethalamic and hippocampal neurons. I appreciate the effort put by the authors in this elegant and thorough work, and I wish more scientists in the field would focus on understanding the poorly characterized mechanisms that drive the differentiation of PSC in vitro.

Comments for the author

I have no essential revisions to suggest. In my opinion, the manuscript is ready for publication.

First revision

Author response to reviewers' comments

We would like to thank the reviewers for their helpful comments on our manuscript. Our responses to the reviewer's comments are below in italics.

Reviewer 1:

Advance Summary and Potential Significance to Field: In this study Medina-Cano and colleagues demonstrate efficient and reproducible differentiation of mouse primed pluripotent stem cells (EpiSCs) into early definitive endoderm and into forebrain organoids. They achieve this by adaptation of protocols developed for human primed PSCs that have some underlying developmental logic. This is of value because although widely ignored, the relatively poor directed differentiation performance of EpiSCs has been a puzzle in the field. Notably, EpiSCs are considered most related to anterior primitive streak and therefore would be expected to differentiate directly and with high efficiency into definitive endoderm in response to developmental signals. The demonstration that this is indeed the case is therefore welcome, as is the finding of consistency between mouse and human primed PSC lineage induction. Although the study does not provide new insights into developmental mechanisms the topic is suitable for the Techniques and Resources section of Development.
However, some additional data are required to consolidate the generality and utility of the findings.

Reviewer 1 Comments for the Author:

Essential revisions:

***The main limitation of the study is that the EpiSCs used are all derived by in vitro conversion of ES cells and cultured in one particular condition. It cannot be certain that embryo-derived EpiSCs will respond equivalently. Therefore, the authors should source embryo-derived EpiSCs and confirm this point.**

We have focused on EpiSC lines that we have derived via in vitro conversion of mouse embryonic stem cells. We chose to do this for the following reasons:

1) *A previous study (Kojima et al., Cell Stem Cell 2014, PMID: 24139757) performed a systematic analysis of EpiSCs derived from different stages of development in vivo as well as mouse ESC-derived EpiSCs. They concluded that EpiSC lines from each of these different sources converge on a highly similar transcriptional program, suggesting that the behavior of distinct lines is determined primarily by the culture conditions rather than the starting cell type. Based on these data, we think it is unlikely that EpiSCs derived directly from embryos will exhibit systematic differences compared to ESC-derived EpiSCs.*

2) *Culture and derivation of mouse ESCs and iPSCs (cultured in naïve conditions) is widespread and there are a large number of transgenic ESC lines available to the community. In contrast, direct derivation of EpiSCs from post-implantation embryos is not commonly performed and far fewer lines exist that have been derived in this manner. Therefore, the vast majority of end users of our protocol would likely be starting with mouse ESCs, and then converting these cells into EpiSCs in vitro.*

While we agree that there is certainly value to a more rigorous analysis of how the derivation conditions of EpiSCs impacts their functional properties and potential to differentiate in vitro, we think that it would be beyond the scope of the current study. We have added the following sentence discussing this in the section in the discussion "Limitations of the current study" (Lines 451-454).

"In this study, we exclusively used EpiSCs (+WI) that we derived in vitro via conversion of naïve mouse PSCs. Previous work suggests that EpiSCs derived in vitro are highly similar to EpiSC lines derived directly from post-implantation epiblast (Kojima et al., 2014), so we would expect cells from both sources to behave similarly, but we have not demonstrated that this is the case."

***Use of feeder layers to maintain the EpiSCs is limiting because they are an unnecessary and potentially confounding factor. It is well established that EpiSCs can be reliably propagated without feeders (or KSR) on fibronectin coated dishes using activin and FGF with or without Wnt inhibition (Guo et al., 2009; Osorno et al., 2012; Tsakiridis et al, 2014). It will be helpful for wider user-friendly adoption of their differentiation protocols that the authors show applicability to feeder-free EpiSCs.**

*In the revised manuscript, we include data demonstrating that we can get similar results with our definitive endoderm differentiation protocol when starting from feeder-free cultures of EpiSCs + Wnt inhibition. These data are presented in **Fig. S3B** of the revised manuscript (see also Lines 197-198).*

We agree that removal of feeders will ultimately be ideal for culturing EpiSCs and differentiating them. We have successfully cultured EpiSCs without feeders on fibronectin or matrigel for up to 10 passages. However, we found that it was more difficult to get consistent growth and passaging in feeder-free conditions across multiple lines from distinct genetic backgrounds, so we do not use this currently as our routine method of EpiSC culture. It is worth noting that extensive optimization was necessary to develop new media formulations for consistent feeder-free culture of human PSCs. Current media formulations used for mouse EpiSC culture have not been optimized to nearly the same extent, and thus it is perhaps not surprising that feeder-free EpiSC cultures are sub-optimal.

***The authors must examine the responsiveness of EpiSCs cultured without Wnt/tankyrase inhibition. They imply throughout that this condition is a pre-requisite but present no evidence whether that is actually the case.**

We did not specifically address this in our manuscript because it has been the subject of multiple previous studies (listed here).

Kurek et al., Stem Cell Reports 2015 (PMID: 25544567)
Sumi et al., Plos One 2013 (PMID: 23691040)
Jun Wu Nature 2015 (PMID: 25945737)
Tsakiridis et al., Development 2014 (PMID: 24595287)

In addition, more recent studies of formative PSCs and primed PSCs similarly suggest that Wnt/Tankyrase inhibition helps to reduce spontaneous differentiation and enables stable long-term culturing of primed PSCs.

Kinoshita et al., Cell Stem Cell 2021 (PMID: 33271069)
Kinoshita et al., Development 2021 (PMID: 34874452)

We have not been able to stably maintain pluripotent EpiSCs in the absence of Wnt inhibition. In contrast, we have been able to readily culture a large number of distinct lines of EpiSCs (n > 100) by including Wnt inhibition.

It is now basically dogma in the hPSC directed differentiation field that the starting population of hPSCs at the beginning of a directed differentiation experiment needs to consist of a high fraction of pluripotent cells with minimal heterogeneity due to spontaneous differentiation. Thus, in our opinion, if we cannot consistently maintain EpiSC cultures in a homogeneous, pluripotent state in the absence Wnt inhibitors, there would be limited value of showing that using these suboptimal, heterogeneous cultures is suboptimal for subsequent differentiation experiments.

***Production of functional definitive endoderm should be validated by further differentiation into e.g. pancreatic or hepatic lineages.**

*In the revised manuscript, we now include proof-of-concept experiments demonstrating that DE generated by our protocol can be further differentiated into posterior foregut and subsequently to progenitors of the developing stomach (antrum). These data are presented in **Fig. 3F,G** and discussed in Lines 226-233 of the revised manuscript.*

***The RNA-seq data on DE differentiation is a useful resource that could be exploited further, in particular for comparative analysis with embryo DE to corroborate identity.**
--It would be helpful to see a global comparison in addition to the selected genes shown in Fig. S2E heatmap.
--It is also not clear how genes shown in the heatmap were selected - have they been cherry picked?

In Figure S2D we compare our data directly to a set of genes identified as being enriched in definitive endoderm from E7.5 mouse embryos using single cell RNA-seq (Nowotschin et al., Nature 2019, PMID: 30959515). For this analysis, we included all of the genes in their gene list -- these genes were not cherry- picked. We did not do a good job explaining this in the original submission and we have revised the manuscript to make it more clear how this gene set was selected (Lines 213-215).

In addition, we also presented an unbiased set of the most variable genes identified across all of our EpiSC and DE samples in Figure S2C.

***It is important to show that the forebrain organoid results are reproducible across EpiSCs of different genetic background, as shown for DE induction. Maybe the authors have done this but it is not apparent in the data presented.**

*In the revised manuscript, we have added a new Supplementary Table (**Table S5**) that includes information about the specific lines used (n = 3) and the number of technical replicates performed for each line (n > 10, n = 2, n = 2 technical replicates).*

***The authors describe their EpiSCs as "primed ground state". I find this terminology unhelpful and potentially confusing to readers. The concept of primed PSCs is well understood in the community but there is neither justification nor criteria presented for use of "ground state" in the present context. "EpiSCs cultured in the presence of Wnt/Tankyrase inhibition" is clear and sufficient.**

We have modified the terminology throughout the manuscript as the reviewer suggests.

Additional suggestions:

***In Figure 1 it would be helpful to include some comparative analysis with ES cells which should show that the EpiSCs are similar to one another and very different from ES cells.**

*We have modified **Figure 1E** to include data from naïve mouse PSCs for the sake of comparison.*

***Have the authors tested less than 16h for aPS induction? If not, they should either do so or else declare they have not done so.**

We have only tested the conditions described in Figure 2 and the associated text (see below).

"To determine the optimal timing and concentration of each signal for aPS induction, we applied a gradient of increasing concentrations of CHIR99201 (to activate Wnt signaling via GSK3 inhibition) and Activin A (to activate TGF-B/nodal signaling) to EpiSCs (DBA/2J) for either 16, 20, or 24 hours." (Lines 165-167)

We have added a note to the revised Figure Legend: "Note: We did not test the effects of < 16 hours of aPS induction." (Lines 722-723)

***Data in Fig 2B do not support the conclusion that a high concentration (100ng/ml) activin is required for maximal DE induction. At 24h with LDN there is little difference over the activin concentration range.**
This is important because 100 ng/ml is not physiological.

In the revised manuscript, we include a sentence describing the rationale for choosing 100 ng/ml Activin A (Lines 183-185) (see below).

"We observed the highest purity of DE differentiation in conditions with the highest levels of Activin A (~75% of cells Sox17+ with 100 ng/mL vs. ~65% Sox17+ cells with 40 ng/mL; Fig. 2B)."

As noted by our reviewing editor Dr. Wells, "100 ng/ml Activin A concentration was first established to promote endoderm differentiation using Xenopus animal cap assays." In addition, 100 ng/ml Activin A is used routinely in the human PSC à definitive endoderm differentiation literature, so our protocol is in line with what is considered the current state of the art for human PSC differentiation.

***The statement naïve PSCs can be converted into stable ground state EpiSC lines within 3-4 days is not accurate. After 4 days cultures will be heterogeneous. Moreover, without serial passaging it cannot be concluded that a stable stem cell population is established.**

We have modified the text in the revised manuscript (Lines 110-111). It now reads "First, EpiSCs (+WI) are easy for labs that currently work with mouse naïve PSCs to acquire, as naïve PSCs can be converted into stable EpiSC (+WI) lines in vitro."

***The statement that EpiSCs potentially have improved karyotypic stability compared to naïve PSCs? is unjustified without evidence (which is not in the cited paper about human PSCs).**

We have removed this statement and reference from the revised manuscript.

***ATAC-seq does not directly map cis-regulatory elements.**

We have modified the language in the revised manuscript to be more precise (see lines 218-224)

***In vivo epiblast is developmentally dynamic from E4-E7.5. Therefore, the authors should declare the stage (E6.5) in the text relating to Fig 1F.**

We have modified the text as suggested by the reviewer (see Lines 141-146).

***The start of the section on DE induction should state that the cells are first transferred to CDM.**

We tried to minimize the number of technical details in the main text for the sake of readability. These experimental details are included in the methods (Lines 504-515) as well as the detailed, step-by-step bench protocols provided with the manuscript. To make this more clear, we have added a call out to the methods in Line 167 of the revised manuscript.

*What is meant by a wild-derived inbred strain??

To reduce confusion, we have removed this term from the main text of the revised manuscript. This is the standard terminology used for strains of mice that were more recently derived from wild-caught populations than the traditional inbred strains used for laboratory research. See e.g.

(https://www.jax.org/strain/003715).

***It is unnecessary and appears rather self-serving to write that the Sasai protocols "were never widely adopted".**

We have removed this phrasing from the revised manuscript.

***In the Introduction section on the requirement for naïve PSC to exit the naïve state to become responsive to inductive cues it would be appropriate to cite Mulas et al., SCR, 2017 which presents direct evidence for this. Similarly, Tsakiridis et al., DEV, 2014 should be cited with regard to WNT pathway inhibition limiting spontaneous differentiation in EpiSC cultures. The subsequent reference to Najm et al. seems misplaced because that paper preceded the WNT pathway discovery.**

We have modified the references as suggested by the reviewer (Line 61 of revised manuscript).

Reviewer 2

Advance Summary and Potential Significance to Field: In this article, Medina-Cano et al. report on the derivation of 12 new EpiSC lines from mESCs. They characterize their stability and their similarity to previous lines and various embryo populations and confirm their similarity to epiblast. They also report on the variability between lines. In a second step they take inspiration from protocols developed for human ESCs and use Wnt and activin and then activin alone to induce definitive endoderm. They carefully optimize their protocol, systematically testing different doses of both pathway activators and the timing on one line. The analysis is quantitative, the conclusion is clear and further tested in two additional lines.
Two rather important things remain unaddressed: (1) what type of endoderm is generated (2) would changes in the dosage and duration of the different growth factors and small molecules improve differentiation in the least efficient line. This would be interesting to know for future users but I don't view this as essential. In the last part, the authors build on the original Eiraku/Sasai protocol to generate forebrain organoids from two of the lines. Based on a combination of markers, the identity of the cells produced is proposed to be forebrain, including dorsal, caudo-medial telencephalon (hippocampus and cortical hem) and dorsal anterior diencephalon (prosomere 3, prethalamus). No comparison with endogenous cells is provided, which is a weakness of the manuscript.

"What type of endoderm is generated?"— We assume the reviewer is asking us to further clarify whether we have generated definitive endoderm rather than primitive endoderm (visceral or

parietal), which share expression profiles of many core marker genes (e.g. Sox17, Foxa2, GATA4/6).

1) *We examined expression of DE marker genes defined from E7.5 scRNA-seq experiments (Fig. S2D). These comparisons suggest that our EpiSC-derived DE cells express numerous genes whose expression is enriched in DE cells in the E7.5 embryo.*
2) *In the revised manuscript, we now include proof-of-concept experiments demonstrating that DE generated by our protocol can be further differentiated into posterior foregut and subsequently to progenitors of the developing stomach (antrum). These data are presented in **Fig. 3F,G** of the revised manuscript (discussed in Lines 226-233 of results section). Given that only DE has the potential to give rise to organ progenitors, we believe provides strong evidence that the cells generated by our protocol are definitive endoderm.*

"Would changes in the dosage and duration of the different growth factors and small molecules improve differentiation in the least efficient line" - This is an interesting question but we have not yet examined this systematically. Based on discussions with the editor, we believe that this would be beyond the scope of the current manuscript.

"No comparison with endogenous cells is provided, which is a weakness of the manuscript." As discussed above, we examined expression of a panel of DE marker genes defined from E7.5 embryos using scRNA-seq in a previous paper (Fig. S2D). This analysis indicates that our EpiSC-derived DE cells express numerous genes whose expression is enriched in DE cells in the E7.5 embryo (Fig. S2D)." For the forebrain organoids, we used the VoxHunt tool (PMID: 3711282) to compare scRNA-seq data from d12 organoids to in situ hybridization data from the Allen Brain Atlas. This analysis was consistent with our immunostaining data (using known markers of progenitors and differentiated neurons from specific regions of the dorsal forebrain) and the cell identities we assigned to clusters in our scRNA-seq datasets (Fig.6H).

In general the work is novel as very little has been done in general in mouse and even less starting from EpiSC. The work is well grounded on developmental principles, which makes the fit to the journal good. The investigation is solid and careful. The manuscript is well written and illustrated. The most important weaknesses of the manuscript are **(1) the feeling of a patchwork associating two different parts, one on endoderm in 2D, only 2 steps of development and one on brain in 3D with many developmental steps; (2) part 1 is conceptually not novel as the molecules and principles used are the same as in human.**

We appreciate the feedback from the reviewer about how the manuscript is framed. Our hope with this manuscript was to demonstrate the potential of EpiSCs (cultured with Wnt inhibitors) as a starting point for directed differentiation experiments. We chose to focus on these two lineages because 1) they are of interest to many investigators, 2) there are efficient and robust protocols available for human PSCs for both lineages, 3) the available mouse ESC protocols for both lineages are limited and thus rarely used in the field.

Reviewer 2
Comments for the Author:

***Page 1: "protocols for directed differentiation of mouse PSCs into defined lineages tend to be slower" What do the authors mean. There have been less people working on such protocols indeed and therefore the field has been slower but as far as I know the protocols are not slower themselves. Yes, the beginning is slower because hES are closer to an epiblast state but then they tend to be slower at later stages. This is actually reported by the authors later in the paper. I don't think the statement is general enough to include in the abstract but the intro is correct.**

We understand why the reviewer was confused here. We have modified the text in the revised abstract to clarify this point (see below).

"Although mice are the most advanced mammalian model system for genetic studies of embryonic development, state-of-the-art protocols for directed differentiation of mouse PSCs into defined lineages require additional steps and generate target cell types with lower purity than analogous

protocols for human PSCs, limiting their application as models for mechanistic studies of development." (Lines 30-33)

***Page 5: (Loh et al., 2014; Morgani and Hadjantonakis, 2020; Yiangou et al., 2018; Zorn and Wells, 2009). There are earlier publications for these two-stage protocols in human comprising a first step of Wnt/activin and then activin alone.**

We added a citation to the following paper (See lines 939-940):

Gadue, P., Huber, T.L., Paddison, P.J., Keller G.M. (2006). Wnt and TGF-B signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc. Natl. Acad. Sci. USA 103, 16806-16811

***Fig 1DE. It is surprising that there are important transcriptional differences between lines derived from B6.129 and C57Bl/6J but little difference by ATAC seq. Do the authors have a tentative explanation?**

We think that the most likely explanations are that this reflects differences in the sensitivity of RNA-seq and ATAC-seq for clustering cell types, or that the differences in ATAC-seq profiles between these lines are not captured by the first two principal components that we plotted for the figure.

***Fig. 5C could be better exploited and linked to the rest of the figure by indicating markers on the map.**

We have modified the figure as suggested.

Note that some references are not complete.

We have fixed the References in the revised manuscript.

***It would be useful to elaborate in the discussion on the differences with the Eiraku protocol (others than the fact it starts from mESCs) that may explain why different brain areas are finally obtained.**

We explain the major differences between our protocol and the Sasai protocol in the discussion (lines 392-400). If we had more space we would include a more detailed discussion but it is difficult to fit in this format.

***I could not find the movies, a pity! As a consequence I could not check them.**

This was perhaps due to a mistake on our part during the process of uploading of the manuscript on the journal submission website. The movies should be available now as part of our revised submission.

Reviewer 3

Advance Summary and Potential Significance to Field: Current protocols for the directed differentiation of mouse PSCs need optimization as they are slower and plagued by poor reproducibility compared to differentiation of hPSC. Unfortunately, so far, the broader developmental biology community has not invested much effort in improving these protocols, limiting our ability to characterize aspects of embryonic development that are not easily accessible and testable in vivo. Here Medina-Cano et al.; established and very thoroughly characterized two protocols for rapid and directed differentiation of mouse epiblast stem that can respectively generate 1) nearly pure definitive endoderm and 2) prethalamic and hippocampal neurons. I appreciate the effort put by the authors in this elegant and thorough work, and I wish more scientists in the field would focus on understanding the poorly characterized mechanisms that drive the differentiation of PSC in vitro.

Reviewer 3 Comments for the Author: I have no essential revisions to suggest. In my opinion, the manuscript is ready for publication.

We appreciate the positive feedback from the reviewer!

Second decision letter

MS ID#: DEVELOP/2022/200561

MS TITLE: Rapid and robust directed differentiation of mouse epiblast stem cells into definitive endoderm and forebrain organoids

AUTHORS: Daniel Medina-Cano, Emily K Corrigan, Rachel A Glenn, Mohammed Tarek Islam, Yuan Lin, Juliet Kim, Hyunwoo Cho, and Thomas Vierbuchen
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.

Reviewer 1

Advance summary and potential significance to field

The authors have responded appropriately to criticisms and suggestions. The Limitations to the Study section includes the key caveats. I am pleased to recommend publication in Development.

Comments for the author

There is a typo in the cell culture section of the Methods: "converted to EpiSCs after 48 hours" should be EpiLCs.

Reviewer 2

Advance summary and potential significance to field

The authors have been responsive to comments and improved the manuscript, which was already quite good. Some requests of reviewer 1 were not fulfilled such as to whether embryo-derived epiSCs would respond similarly to the differentiation as ESC-derived epiSCs but the authors provide relatively good arguments.
Among improvements, proof of further differentiation of endoderm into gastric antrum is shown (new figure panels 3E)(rather than the requested liver and pancreas). Former Figure S2 was split into two figures, enabling to document the newly explored and less reliable feeder-free conditions (Figure S3B).

Comments for the author

However, to my question “What type of endoderm is generated? the authors respond:
”— We assume the reviewer is asking us to further clarify whether we have generated definitive endoderm rather than primitive endoderm (visceral or parietal), which share expression profiles of many core marker genes (e.g. Sox17 Foxa2, GATA4/6).
1) We examined expression of DE marker genes defined from E7.5 scRNA-seq experiments (Fig. S2D). These comparisons suggest that our EpiSC-derived DE cells express numerous genes whose expression is enriched in DE cells in the E7.5 embryo.”
It would be a lot more valuable to include DE samples in the comparison. Comparing the expression of DE markers between epiSC and epiSC-derived DE Indeed shows that epiSC-derived DE converges

towards DE but does not address how similar to DE it is. It may be difficult to do well at this point if they use a previously published dataset performed with a different methodology.
In addition to the new ref. to Gadue, I would recommend to add https://doi.org/10.1038/nbt1259, which was published at the same time and also uses a combination of Wnt and activin.
Primed ground state is still used in the title of Figure 1

Reviewer 3

Advance summary and potential significance to field

The authors have satisfactorily addressed the concerns raised in the last manuscript version by the other reviewers. Therefore, I confirm the previous recommendation and support the publication of this critical study for the field.

Comments for the author

The authors have satisfactorily addressed the concerns raised in the last manuscript version by the other reviewers. Therefore, I confirm the previous recommendation and support the publication of this critical study for the field.