Molecular divergence of mammalian astrocyte progenitor cells at early gliogenesis

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Original submission

First decision letter

MS ID#: DEVELOP/2021/199985

MS TITLE: Molecular Divergence of Mammalian Astrocyte Progenitor Cells at Early Gliogenesis

AUTHORS: Qiang Lu, Jiancheng Liu, and Xiwei Wu

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 identification of two distinct astrocyte precursor populations (ACP) during gliogenesis, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. Both referees ask for additional information, quantification and details of the methods to help strengthen your observations. In addition, including data assaying SPARC and SPARCL1 staining with at least one other marker that discriminates the two subsets would be a clear validation of your single cell data. I would also draw your attention to Referee 2's suggestion to check ependymal gene expression markers (eg. Foxj1) to determine whether on of the ACPs is related to ependymal cells.

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.

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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, the authors used single cell sequencing to define embryonic mouse forebrain subpopulations.
While recent studies have begun to identify distinct subpopulations of astrocytes with specific functions, this study aims to determine if astrocyte heterogeneity can be defined during specification. These authors identified two distinct astrocyte precursor populations (ACP) during gliogenesis. One population is marked by the expression of Sparc while the second is marked by Sparcl1. Spatial-temporal expression of these markers in embryonic brain populations was validated using immunofluorescence techniques. These findings are of interest to developmental and glial biologists as markers that define specific astrocyte populations are generally lacking. Given that this manuscript is a descriptive characterization of cellular populations there seems to be a lack of robustness overall (see comments below) and writing of the manuscript.

Comments for the author

There are several revisions that would benefit the manuscript:
1. In general the introduction is lacking in background information which reflects a less than thorough knowledge of the field. For example, several high profile studies related to this manuscript are overly summarized, not discussed or not cited (including Lin 2017, Weng 2019 cell stem cell, Marques 2016).
Some of these studies directly address glial precursor cell diversity.
2. The rationalization for using Nestin-GFP vs other potential reports is not clearly justified. The authors should clearly define what differentiates this manuscript from previous studies.
3. Performing a Developmental Trajectory Analysis on their single cell data would strengthen their analysis and better demonstrate developmental dynamics of the developmental populations isolated, especially as they claim that the SPARC+ and SPARCL1+ populations might be generated in a temporal sequence (pg12)
4. FACS plots documenting the purification of NPCs should be included in the supplemental figures to strengthen the argument.
5. There seems to be a typo in the differentially expressed genes between the two ACP subgroups in the cortex section. In the fourth line the text is referring to S3A not 3A.
6. Showing Sparc and Sparcl1 co-staining would strengthen the argument that these two genes are spatially and temporally distinct populations. I realize that the antibodies used were both goat but RNAscope (or in situ) probes could be used to show that these are separate populations.
7. There is a lack of quantification in all the figures that claim co-localization of Sparc and Sparcl1 with other markers. For example, in Figure 4, it appears that Aldh1l1-GFP positive cells are not 100% co-positive with either Sparc and Sparcl1. In another example, Figure 3 quantification of Sox9 double positive cells is lacking. Or in Figure 2 with Pax6. In the text pg9 the authors say that ki67 and sparcl1 are coexpressed in 40% of cells however, the graphical representation of this data with statistics is not provided. Again, in general the inclusion of quantification is lacking in the document.
8. Putting the data in the context of previous studies should be included in the discussion section.
9. In general, there is a lack of details in the methods. The details of the experiments are not described.
While referencing a paper is acceptable, at the very least this should be followed by "in brief... [description]". This is relevant to several of the method descriptions including purification of NPCs and immunohistochemistry. In addition, there needs to be consistency in nomenclature, as in CD31 and PCAM are used interchangeably.
10. There are several grammatical errors that could be addressed to improve the readability.

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11. There are no heading in the paper to distinguish between intro, results, and discussion. This should be added to improve readability.

Reviewer 2

Advance summary and potential significance to field

Using single cell RNA-seq of Nestin-GFP+ cells of the developing telencephalon the authors discover 2 spatially restricted progenitor population that they associate to the astrocyte lineage based on co-localization with Aldh1l1-GFP. Intriguingly, these two subsets can be discriminated by SPARC versus SPARCL1 expression and immunostaining and localize in distinct domains. Based on expression analysis and immunostainings at different stages the authors propose that the SPARCL1-population could give rise to the proliferating astrocyte progenitors in the postnatal SVZ, while SPARC+ progenitors would give rise to astrocytes by other means (e.g. translocation from VZ). As the authors neither include live imaging in slice preparations nor genetic fate-mapping to substantiate this indeed very intriguing possibility, this remains to be seen. However, the identification of these subpopulations and their spatial distribution may already be of sufficient interest to the developmental community, as it provides an intriguing novel marker and hypothesis.

Comments for the author

1) Generally, the author provide micrographs of too low magnification to adequately judge co-localization of immunostainings. Please show high magnifications with only 1-2 cells per field and a Z-projection of a confocal stack to ensure colocalization in the cells.
2) To verify the subtype populations the authors should combine SPARC and SPARCL1 staining with at least 1 other protein that discriminates the APC1 and APC2 subset. For example, there is good Zac1 (Plagl1) antibodies and this is expressed higher in the APC1 subset, or Brevican and Id1-3 antibodies that should label the SPARCL1 APC2 subset.
3) The postnatal analysis with the SPARCL1 emigrating cells is particularly interesting, but requires quantification – how many of the SPARCL1 non-vascular cells are in the SVZ and Ki67+ and how many in the differentiating cortex layers in order to corroborate if there is really enrichment in the SVZ.
4) The authors call these cells astrocyte progenitors and do not consider ependymal cells and their progenitors at all. Ependymal cells have all astrocyte “markers”, just to different levels, and the high ApoE and low GLAST expression is reminiscent of ependymal cells. Please check ependymal gene expression from various recently published scRNA-seq papers as well as Foxj1, McIDAS and Lynkeas genes that are important for ependymal cell differentiation around E18 when the APC1 and APC2 subclusters are detected.
Could it be that these are ependymal progenitors that later may give rise to few astrocytes? Could it be that the latter are the region-specific astrocyte subtypes as they stay local, while the SPARCL1-derived astrocytes are the less regionalized astrocytes performing pan-astrocyte functions?

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
In this study, the authors used single cell sequencing to define embryonic mouse forebrain subpopulations. While recent studies have begun to identify distinct subpopulations of astrocytes with specific functions, this study aims to determine if astrocyte heterogeneity can be defined during specification. These authors identified two distinct astrocyte precursor populations (ACP) during gliogenesis. One population is marked by the expression of Sparc while the second is marked by Sparcl1. Spatial-temporal expression of these markers in embryonic brain populations was validated using immunofluorescence techniques. These findings are of interest to developmental and glial biologists as markers that define specific astrocyte populations are generally lacking.

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Given that this manuscript is a descriptive characterization of cellular populations there seems to be a lack of robustness overall (see comments below) and writing of the manuscript.

We thank reviewer for the positive comment on the impact of this study.

Reviewer 1 Comments for the Author:
There are several revisions that would benefit the manuscript:
1. In general the introduction is lacking in background information which reflects a less than thorough knowledge of the field. For example, several high profile studies related to this manuscript are overly summarized, not discussed or not cited (including Lin 2017, Weng 2019 cell stem cell, Marques 2016). Some of these studies directly address glial precursor cell diversity.

Thanks for pointing out this oversight. The Lin 2017 paper was cited in our manuscript, however, Endnote list mistakenly referred to it as John Lin 2017. We will need to correct this mistake at the stage of galley proofing, so to avoid any potential unintended errors into the Endnote file.
Characterization of juvenile and adult glial progenitor cells by Weng 2019 and Marques 2016 were included (Pages 4 and 10).

2. The rationalization for using Nestin-GFP vs other potential reports is not clearly justified. The authors should clearly define what differentiates this manuscript from previous studies.

Our previous characterization of transcriptomes of pooled NPCs and the current study of single NPCs isolated from the combination of Nestin/Dcx dual reporter mice indicated that the Nestin-GFP reporter marks most, if not all, types and states (cell cycle states) of NPCs in the developing mouse forebrains. What we did uniquely in this study was that we focused our scRNA-seq analyses on the NPC population (enriched from the brains) and compared two developmental stages at the neurogenesis-to-gliogenesis transition. We believe that these two aspects in our approach helped reveal distinct NPC subtypes and their respective molecular signatures with confidence. We included this discussion in the introduction on pages 4 and 5.

3. Performing a Developmental Trajectory Analysis on their single cell data would strengthen their analysis and better demonstrate developmental dynamics of the developmental populations isolated, especially as they claim that the SPARC+ and SPARCL1+ populations might be generated in a temporal sequence (pg12)

As suggested, we have performed a developmental trajectory analysis. The result is included as Fig. 3D-F, which showed SPARC+ cells might be generated earlier than SPARCL1+ cells, consistent with our immunostaining and RNAscope expression analyses.

4. FACS plots documenting the purification of NPCs should be included in the supplemental figures to strengthen the argument.

FACS profiles of NPC isolation at E15.5 and E18.5 stages were included as Supplementary Fig. S1.

5. There seems to be a typo in the differentially expressed genes between the two ACP subgroups in the cortex section. In the fourth line the text is referring to S3A not 3A.

Thank you for pointing out this mistake. We have corrected it.

6. Showing Sparc and Sparcl1 co-staining would strengthen the argument that these two genes are spatially and temporally distinct populations. I realize that the antibodies used were both goat but RNAscope (or in situ) probes could be used to show that these are separate populations.

Yes, the antibodies were both from goat, so prohibited a co-staining. As suggested, we have done a co-staining with RNAprobe, however, co-staining of one RNAscope and one antibody did not work since neither SPARC nor SPARCL1 antibody could work well on heat-treated tissues. We therefore did co-staining with two RNAscope probes. The results are included in Fig. 3A-C, showing clearly that the two groups of cells are spatially segregated. The new data are also

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consistent with antibody staining patterns.

7. There is a lack of quantification in all the figures that claim co-localization of Sparc and Sparcl1 with other markers. For example, in Figure 4, it appears that Aldh1l1-GFP positive cells are not 100% co- positive with either Sparc and Sparcl1. In another example, Figure 3 quantification of Sox9 double positive cells is lacking. Or in Figure 2 with Pax6. In the text pg9 the authors say that ki67 and sparcl1 are coexpressed in 40% of cells however, the graphical representation of this data with statistics is not provided. Again, in general the inclusion of quantification is lacking in the document.

Quantifications were included in new figures: Fig. 2C (Pax6), Fig. S6C (Sox9) and Fig. S8A (Aldh1l1-GFP reporter), S8C (Ki67).

8. Putting the data in the context of previous studies should be included in the discussion section.

We have revised our discussions to our best knowledge.

9. In general, there is a lack of details in the methods. The details of the experiments are not described. While referencing a paper is acceptable, at the very least this should be followed by "in brief... [description]". This is relevant to several of the method descriptions including purification of NPCs and immunohistochemistry. In addition, there needs to be consistency in nomenclature, as in CD31 and PCAM are used interchangeably.

We have revised methods section with more detailed descriptions (page 14 and 16). As suggested, PECAM1/CD31 was used in all relevant figure legends and in most figure panels.

10. There are several grammatical errors that could be addressed to improve the readability.

We have tried our best to correct any grammatical errors.

11. There are no heading in the paper to distinguish between intro, results, and discussion. This should be added to improve readability.

The Research Report format requires Results and Discussions be combined as one section, thus we did not mark Results and Discussions with separate headings. We now revised to use Results and Discussions as a combined title (page 5).

Reviewer 2 Advance Summary and Potential Significance to Field:
Using single cell RNA-seq of Nestin-GFP+ cells of the developing telencephalon the authors discover 2 spatially restricted progenitor population that they associate to the astrocyte lineage based on co- localization with Aldh1l1-GFP. Intriguingly, these two subsets can be discriminated by SPARC versus SPARCL1 expression and immunostaining and localize in distinct domains. Based on expression analysis and immunostainings at different stages the authors propose that the SPARCL1-population could give rise to the proliferating astrocyte progenitors in the postnatal SVZ, while SPARC+ progenitors would give rise to astrocytes by other means (e.g. translocation from VZ). As the authors neither include live imaging in slice preparations nor genetic fate-mapping to substantiate this indeed very intriguing possibility, this remains to be seen. However, the identification of these subpopulations and their spatial distribution may already be of sufficient interest to the developmental community, as it provides an intriguing novel marker and hypothesis.

We thank reviewer for the positive comment on the impact of this study.

Reviewer 2 Comments for the Author:

1) Generally, the author provide micrographs of too low magnification to adequately judge co-localization of immunostainings. Please show high magnifications with only 1-2 cells per field and a Z- projection of a confocal stack to ensure colocalization in the cells.

High mag confocal images were added in Fig. 3C, Fig. 4D and 4F. Of a note, as SPARC and SPARCL1 are secreted proteins, their co-expression with cellular markers, such as Aldh1l1-GFP

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reporter, might not conform to co-expression patterns between intracellular proteins. But overall, staining signal of SPARC or SPARCL1 was seen overlapping or closely associated with individual GFP positive cells.

2) To verify the subtype populations the authors should combine SPARC and SPARCL1 staining with at least 1 other protein that discriminates the APC1 and APC2 subset. For example, there is good Zac1 (Plagl1) antibodies and this is expressed higher in the APC1 subset, or Brevican and Id1-3 antibodies that should label the SPARCL1 APC2 subset.

As suggested, we included CLU staining (SPARC group APCs) (Fig. S5D). In addition, we performed co-staining of Sparc and Spacrl1 using RNAprobe (Fig. 3A-C).

We have also tested anti-ID3 (Abcam, ab41834), anti-PTX3 (Enzo, ALX-210-365-C050), anti-ZAC1(PLAGL1) antibody (Santa Cruz, sc-166944), anti-BCAN (Proteintech, 19017-1-AP), as well as rabbit anti-SPARC (Proteintech, 15274-1-AP) and rat anti-SPARC (R&D System, MAB942). However, none of these antibodies could work well for tissue staining.

3) The postnatal analysis with the SPARCL1 emigrating cells is particularly interesting, but requires quantification – how many of the SPARCL1 non-vascular cells are in the SVZ and Ki67+ and how many in the differentiating cortex layers in order to corroborate if there is really enrichment in the SVZ.

The potential lateral-to-medial dispersion of SPARCL1$^+$ APCs from E16.5 to P1 was indeed quite intriguing. The proliferating (Ki67$^+$) APCs were located at the apical surface of the ventricle and their numbers (overall numbers of SPARCL1$^+$ APCs as well) decreased significantly around the neonatal stage. These temporal changes made it hard to analyze proliferating SPARCL1$^+$ APCs in the PSB (SVZ) area of postnatal brains. To help reveal the apparent lateral-to-medial dispersion of SPARCL1$^+$ APCs, we included SPARCL1 staining patterns at E17.5 and E19.5 (Fig. S9), which together with data in Fig. 2D, showed the temporal progression of cell distributions as well as depletion of the proliferating pool. Future experiments with Sparcl1-specific Cre lines and live cell imaging of brain slices will be necessary for better investigating into this issue.

4) The authors call these cells astrocyte progenitors and do not consider ependymal cells and their progenitors at all. Ependymal cells have all astrocyte “markers”, just to different levels, and the high ApoE and low GLAST expression is reminiscent of ependymal cells. Please check ependymal gene expression from various recently published scRNA-seq papers as well as Foxj1, McIDAS and Lynkeas genes that are important for ependymal cell differentiation around E18 when the APC1 and APC2 subclusters are detected. Could it be that these are ependymal progenitors that later may give rise to few astrocytes? Could it be that the latter are the region-specific astrocyte subtypes as they stay local, while the SPARCL1-derived astrocytes are the less regionalized astrocytes performing pan-astrocyte functions?

In our scRNA-seq data of E18.5 NPCs, ependymal genes, such as Foxj1, Mcidas, and Gmnc/GemC1/Lynkeas, were not detected in high level in any of the 10 clusters of cells (please see below the violin plots of representative genes). Thus, the pool of NPCs purified from the E18.5 brains appeared to contain few or no ependymal lineage cells at this stage. Based on the protein and RNA staining patterns, it appeared more likely that SPARC-APCs were originated from transforming radial glia cells, while SPARCL1-APCs might be generated separately. Future cell fate mapping using Sparc and Sparcl1 specific Cre lines would be necessary to further define these two astrocyte lineages.

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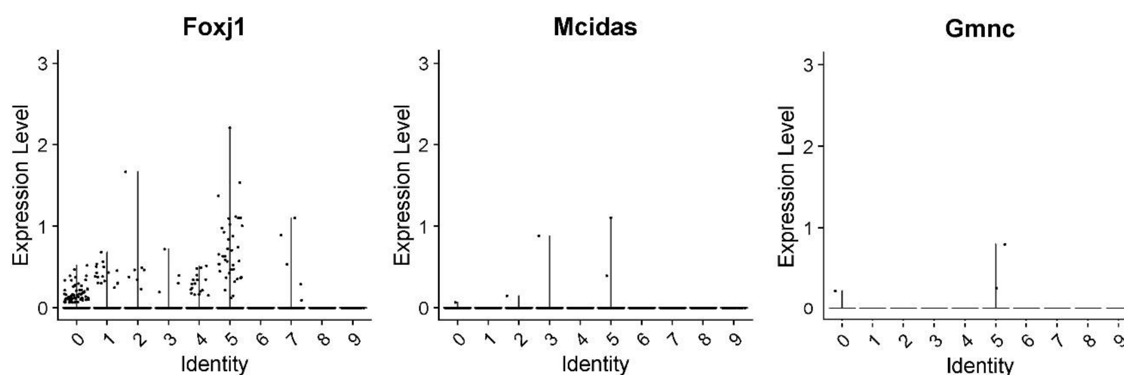

Second decision letter

MS ID#: DEVELOP/2021/199985

MS TITLE: Molecular Divergence of Mammalian Astrocyte Progenitor Cells at Early Gliogenesis

AUTHORS: Qiang Lu, Jiancheng Liu, and Xiwei Wu

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 reviewers recognise the improvements made to the study and we would like to publish a revised manuscript in Development, however this requires satisfactorily addressing the referees' comments. In particular, Reviewer 2 raises three issues that need to be addressed. Improving the quality of the Sox9 imaging to test for colocalisation and performing Clu/SPARC costaining appear to be important. 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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

In this study, the authors used single cell sequencing to define embryonic mouse forebrain subpopulations. While recent studies have begun to identify distinct subpopulations of astrocytes with specific functions, this study aims to determine if astrocyte heterogeneity can be defined during specification. These authors identified two distinct astrocyte precursor populations (ACP) during gliogenesis. One population is marked by expression of Sparc while the second is marked by Sparcl1. Spatial-temporal expression of these markers in embryonic brain populations was validated using immunofluorescence techniques. These findings are of interest to developmental and glial biologist. The authors have appropriately addressed my concerns.

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Comments for the author

The authors have addressed my concerns.

Reviewer 2

Advance summary and potential significance to field

The authors identified distinct glial progenitor cell clusters expressing SPARC and SPARCL1 respectively. These could provide useful markers and delineate at an early stage distinct glial subtypes.

Comments for the author

Unfortunately the authors did a very superficial revision, and still do not provide convincing high power Z-projection images for some of their co-localization. For example, they state on p. 8 that 96% of SPARCL1+ cells were Sox9+. However, the stainings shown in S6B are low power, and most red SPARCL1-staining is next to the green nuclei, but not surrounding them as visible in these low mag pictures. This claim of colocalisation with Sox9 is simply not justified by the data shown. Moreover, we have no information how many cells were quantified (N=3 should mean in 3 different animals, but what is the n?).
Likewise stainings shown in S8 are low power and the dotty stains do not justify at all the allegedly 98% co-localization.
Also Clu staining (Figure S5D) is shown without any double-labelling, just labelling all the cells at the ventricle, but if there is co-localisation with SPARC or not is not shown.
I am also not convinced that SPARC+ cells would not be the ancestors of ependymal cells, given the Clu+ cell lining at the ventricle and the high expression of Clu in this cluster. Maybe McIDAS etc markers are not yet expressed at this stage in these cells, but this possibility should AT LEAST be mentioned and discussed.

Second revision

Author response to reviewers' comments

Reviewer 2 Comments for the Author:
Unfortunately the authors did a very superficial revision, and still do not provide convincing high power Z- projection images for some of their co-localization. For example, they state on p. 8 that 96% of SPARCL1+ cells were Sox9+. However, the stainings shown in S6B are low power, and most red SPARCL1-staining is next to the green nuclei, but not surrounding them as visible in these low mag pictures. This claim of colocalisation with Sox9 is simply not justified by the data shown. Moreover, we have no information how many cells were quantified (N=3 should mean in 3 different animals, but what is the n?).
Likewise stainings shown in S8 are low power and the dotty stains do not justify at all the allegedly 98% co- localization.

We have revised Fig. S6B to include a high magnification of co-staining of SPARCL1 and SOX9 to document co-expression of SPARCL1 and SOX9 in many cells of the embryonic brains. As we discussed in the previous Response, because SPARCL1 and SPARC are secreted proteins, co-staining with other intracellular markers might not conform to the co-expression patterns seen for two intracellular factors with confocal images. The new high mag picture in Fig. S6B showed that SPARCL1 staining was closely associated with identifiable cells (by Hoechst) demarcated by SOX9 staining, a pattern consistent with SPARCL1 being secreted into the extracellular matrix of parental cells.

In addition, we have included the average n (from N=3 brains) in the legends of Fig. S6C (page 9 of Supplementary Figures), Fig. S8A and S8C (page 12 of Supplementary Figures).

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Also Clu staining (Figure S5D) is shown without any double-labelling, just labelling all the cells at the ventricle, but if there is co-localisation with SPARC or not is not shown.

Unfortunately, the available CLU antibody working for IHC was also from goat, the same species as the SPARC antibody (information about the antibodies was discussed in Methods section of the manuscript). As we discussed in the previous Response, we had also tested a number of other antibodies, including a rabbit anti-SPARC (Proteintech, 15274-1-AP) and rat anti-SPARC (R&D System, MAB942). However, neither antibody could work well for tissue staining, thus preventing a co-staining with CLU antibody. Despite this, the current data with CLU staining was consistent with our scRNA-seq data which suggested that SPARC and CLU are expressed by group 1 APCs. Future available antibodies or genetic reporter strains will help further validate this co-expression.

I am also not convinced that SPARC+ cells would not be the ancestors of ependymal cells, given the Clu+ cell lining at the ventricle and the high expression of Clu in this cluster. Maybe McIDAS etc markers are not yet expressed at this stage in these cells, but this possibility should AT LEAST be mentioned and discussed.

We agree that the lack of Foxj1 or Mcidas expression in cell clusters of our scRNA-seq data did not exclude the possibility that the SPARC and SPARCL1 cells may generate other glial cell types such as ependymal cells. We have included a discussion in the manuscript to discuss about this possibility (page 12-13).

Third decision letter

MS ID#: DEVELOP/2021/199985

MS TITLE: Molecular Divergence of Mammalian Astrocyte Progenitor Cells at Early Gliogenesis

AUTHORS: Qiang Lu, Jiancheng Liu, and Xiwei Wu
ARTICLE TYPE: Research Report

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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