nature portfolio

Peer Review File

Developmental Chronology of Mouse Embryo from 2-cell stage through birth

Corresponding Author: Professor Duanqing Pei

Version 0:

Decision Letter:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Professor Pei,

I apologize for the delay as we had previously experienced difficulty in securing sufficient reviewer expertise to comment on your submission.

Your manuscript, "Developmental Chronology of Mouse Embryo", has now been seen by 2 referees, who are experts in mammalian embryonic development (referee 1); single cell atlases (referee 2). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A) Demonstrate the advance and utility presented by your resource through comparisons with previously published literature including your reference [10] (Qiu et al 2024) (Reviewer #1)

B) Provide further analyses regarding any such claims presented about differential expressed genes and address all concerns about such analyses (both Reviewers)

C) All other referee concerns pertaining to strengthening existing data, providing controls, methodological details, clarifications and textual changes, should also be addressed.

D) Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

- for any revision that includes light microscopy data, we ask our authors to please include a completed light microscopy reporting table https://www.nature.com/documents/Light_microscopy_reporting_table.xlsx to ensure the methods are described thoroughly. The table will be available to reviewers and ultimately published should the manuscript be accepted at the journal.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and www.nature.com/nature/authors/).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Editorial Policy Checklist (found here https://www.nature.com/authors/policies/Policy.pdf), and Reporting

Summary (found here https://www.nature.com/authors/policies/ReportingSummary.pdf). This is essential for reconsideration of the manuscript and these documents will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

Nature Cell Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

Link Redacted

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months. We would be happy to consider a revision even after this timeframe, however if the resubmission deadline is missed and the paper is eventually published, the submission date will be the date when the revised manuscript was received.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Daryl

Daryl Jason Verzosa David, PhD

Senior Editor, Nature Cell Biology
Advisory Editor, npj Biological Physics and Mechanics
Nature Portfolio

Heidelberger Platz 3, 14197 Berlin, Germany
Email: daryl.david@nature.com
ORCID: https://orcid.org/0000-0002-9253-4805

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

Cao, Lin, Feng, Chen, Wu et al.'s manuscript titled "Developmental Chronology of Mouse Embryo" builds a comprehensive single cell atlas of mouse embryology from the ~2 cell stage through to birth. By unifying their data collection by assay (the 10x Chromium NextGEM v3 3' UTR), they have built a robust reference comprised of ~2.2 million cells using a methodology that generally provides higher per cell transcript recovery and minimal batch artifacts, two features that can hamper high resolution analysis of differential gene expression, lineage trajectory, or cell type annotation. They then utilize this resource to highlight a number of compelling features of embryogenesis, including the comparatively delayed differentiation of epithelial subtypes (predominantly within the endoderm) as well as the ability to identify and test master regulators of key lineages (the pituitary gland TF Tub, conditional knockout of which leads to diminished gonadotrope abundance and obesity).

Overall, the authors are correct in highlighting some of the current limitations in developmental atlas building, which in the absence of a pure assay lock and consistent techniques can lead to integration artifacts that limit their utility. Similarly, per cell transcript recovery can diminish one's ability to identify rare subtypes or to distinguish gene regulatory patterns associated with differentiation. As a resource, the data collected by the Center for Cell Lineage and Atlas group has a compelling case to make and I do not doubt it would be utilized by the field. However, given the lack of technological or analytical novelty within this manuscript, I think the authors could do more to demonstrate the value of their resource in comparison to other approaches, as well as to clarify the actual improvements that they have made. At the moment, many of the reported improvements are "showcased" (eg. by showing markers or clustering with their data versus published data slices) rather than rigorously analyzed in a unified setting. I think this does a disservice to the resource and the field overall, as it is often hard to tell how many of the reported benefits stem from comparing a custom data set to a public one, rather than reanalyzing that repository for the purposes of a fair comparison.

In particular, to me it is somewhat difficult to assess from the methods how actively versus passively the authors compared their dataset to that of the Shendure group (Qiu et al., 2024, reference 10), which could bias the interpretation that the data is itself better for these purposes. In general, I ask that the authors work to unify both data sets for the purposes of their analysis and argument. For example, if the authors claim that the improved transcript recovery is responsible for their ability to more precisely define cell types, they should process and analyze the Qiu data in the same manner: meaning, perform de novo clustering on equivalent subsections of the data (portions of the data that represent equivalent subpopulations), and then demonstrate the improved precision of their data set in

identifying novel clusters (cell types) in comparison to the Qiu et al. data set using the same or similar parameters. Alternatively, the authors could elect to integrate the Qiu et al data to their own reference, which would allow them quantitatively showcase the relative expression of marker genes between the two data sets in a unified manner (meaning, they can show the cluster-level expression of variable or marker genes for every cluster between the two data sets using the same labels). The methods state that the authors have downloaded and revisualized the Qiu data using Scanpy, so either option should not be too challenging to implement and include as part of the extended data.

From the same point of view, I think it is equally important that the authors explicitly show how their decision to dissect and sample distinct tissue compartments may bias their data set versus Qiu et al, which I believe sampled nuclei from entire embryos without dissection or tissue specific digestion/processing. The authors should use this prior data set to state at what resolution their work can make accurate/unbiased statements about cell state composition within a developing embryo, for example by comparing matched time points and/or individual tissue systems to the Shendure group data, and then calculating the relative divergence (distance) between them. It may be possible that the Shendure data set and this data set diverge at the level of the full embryo, but that they are more comparable within specific subcompartments (epithelium, brain, or other). These types of analyses would be best conducted on unified cell state labels as requested above.

Similarly, the key benefit of combinatorial indexing (the Shendure group's method) is that it can sample a larger number of cells per embryo at a reduced per cell transcript count. Here, I would like to see the authors include as extended data comparable estimates of the per embryo "coverage" between the two groups (the fraction of cells sequenced per embryo as a function of developmental time), even if they must proceed from published estimates of the number of cells per embryo stage. I am particularly surprised at how visually similar the cell coverage appears to be when the authors compare the two atlases for different compartments (for example in Figure 2 as well as ED Figures 3 and 4), particularly given the larger number of cells that Qiu et al sampled overall. As a minor comment, the authors should include n's on these plots to highlight the number of cells included, and be more explicit about which timepoints these cells are derived from in both data sets.

I am also curious about the author's explanation that the larger number of DEGs reflects higher per cell transcriptome coverage and ask for more analysis to this point. The combinatorial indexing strategies utilized by Qiu et al sample much larger cell numbers (~11M to 2M cells, a roughly 5-fold difference) with only an ~2-3 fold decrease in the number of recovered transcripts per cell. In general, my expectation would be that lower per cell transcript sampling would be compensated by higher numbers of sampled cells, but this doesn't appear to be true in the data presented in Figure 2 or Extended Data Figures 3 and 4, which show striking disparities in the expression of the same marker genes. These results suggest that no amount of additional sampling would adequately recover an accurate "pseudobulk" based assessment of a given cell state's transcriptome. Here, I would ask that the authors demonstrate the degree to which this may or may not be true by showing the relative correlation between normalized pseudobulk-level transcript counts between the two data sets on unified labels, both for the total transcriptome and for marker genes, and to test in certain cases whether or not this correlation is stable as a function of sampled cells.

Finally, although this manuscript is being considered as a Resource, it is worth highlighting that two novel descriptive findings using this data (the terminal differentiation of metanephric progenitors or the synchronized late timing of epithelial/endodermal differentiation) are not validated with additional in situ data, such as in situ hybridization or immunofluorescence. While this may not be a major requirement for a Resource article, the authors do state that their methodology allows them to discover these previously unobserved processes, so some additional validation that these markers are present in the tissues with the dynamics described seems worth considering for inclusion in a revised manuscript.

Reviewer #2 (Remarks to the Author):

This study presents a comprehensive single-cell transcriptomic atlas that offers a detailed view of mouse embryonic development from E1.5 to E19.0. The key strengths of this work include: (1) exceptional data breadth and depth, covering 37 developmental time points and approximately 2 million cells; (2) high data quality in terms of gene detection and cell-type annotation, outperforming existing atlases (e.g., Qiu et al., 2024); and (3) identification of two distinct nephron progenitor cell (NPC) populations with intriguing and non-overlapping temporal dynamics. The resulting mdCLA atlas represents a valuable resource for the developmental biology community. However, several aspects require further attention to enhance the impact and clarity of the study.

Major Issues

1. While the dataset is rich, the analyses presented are relatively superficial and do not fully exploit its potential. For example, are NPC1 and NPC2 developmentally related? Their non-overlapping temporal windows (E13–E15) suggest distinct origins. This relationship could be explored computationally using tools such as pseudotime analysis or RNA velocity, which may reveal lineage trajectories and clarify whether these populations arise from a common precursor or independent lineages.
2. The concept of "accelerated differentiation" in epithelial cells (Figure 3) is intriguing but lacks robust validation. Trajectory inference using methods such as Slingshot, Monocle 3, or Palantir would provide stronger support for this claim. In particular, the optimal transport-based inference shown in Figure 3B is insufficiently explained — both the methodology and rationale should be clarified, ideally with benchmarking against more conventional trajectory tools.
3. A time-resolved analysis of gene regulatory networks (GRNs) or transcription factor (TF) modules could significantly enhance the study. For instance, in the pituitary epithelial lineage, Tub is known to play a role in pituitary function. The current analysis should either: demonstrate how mdCLA independently recapitulates Tub's known functon(e.g., through stage-specific GRNs or temporal expression dynamics), or shift emphasis to atlas-driven discoveries (e.g., identification of novel TFs enriched in specific pituitary epithelial subtypes).
4. As this is primarily a resource paper, the authors should consider providing a more user-friendly interface for data access and visualization. This could include a searchable web portal, download-friendly metadata tables, and precomputed gene expression plots to facilitate broad reuse by the community.

Minor Issues

1. While the authors identify two NPC populations, their developmental origins and fates remain unclear. Is NPC2 derived from NPC1, or do they emerge from separate progenitors? Additionally, are these populations associated with distinct nephrogenic niches? The mention of immune-related GO terms in NPC2 is intriguing — could this suggest a microenvironmental influence or signaling niche that has not yet been characterized?
2. Some figures require better explanation, especially Figure 3B (trajectory tree) and Figure 3C (subtype timing). The current figure

legends and main text descriptions are insufficient to interpret these panels independently.

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

MANUSCRIPT FORMAT – please follow the guidelines listed in our Guide to Authors regarding manuscript formats at Nature Cell Biology.

TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

AUTHOR NAMES – should be given in full.

AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see https://www.nature.com/licenceforms/nrg/competing-interests.pdf.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled “Statistics and Reproducibility”.

All Nature Cell Biology manuscripts submitted on or after March 21 2016 must include a Data availability statement at the end of the Methods section. For Springer Nature policies on data availability see http://www.nature.com/authors/policies/availability.html; for more information on this particular policy see http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf. The Data availability statement should include:

• Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For

primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here http://www.nature.com/authors/policies/availability.html#data.

• Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details http://www.nature.com/sdata/data-policies/repositories).

• At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.

• If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

We recommend that you upload the step-by-step protocols used in this manuscript to protocols.io. More details can found at https://www.protocols.io/help/publish-articles.

DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

FIGURES – Colour figure publication costs $600 for the first, and $300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend.
Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.

- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the “unprocessed scans” Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial http://www.nature.com/ncb/authors/submit/index.html#suppinfo; http://www.nature.com/ncb/journal/v14/n3/index.html#ed). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – To improve the quality of methods and statistics reporting in our papers we have recently revised the reporting checklist we introduced in 2013. We are now asking all life sciences authors to complete two items: an Editorial Policy Checklist (found here https://www.nature.com/authors/policies/Policy.pdf) that verifies compliance with all required editorial policies and a reporting summary (found here https://www.nature.com/authors/policies/ReportingSummary.pdf) that collects information on experimental design and reagents. These documents are available to referees to aid the evaluation of the manuscript. Please note that these forms are dynamic ‘smart pdfs’ and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use by the reviewers. If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from n<3. For sample sizes of n<5 please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled “Statistics Source Data”, and mentioned in all relevant figure legends.

--------- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements ---------

Version 1:

Decision Letter:

Our ref: NCB-RS58009A

24th February 2026

Dear Dr. Pei,

Thank you for submitting your revised manuscript "Developmental Chronology of Mouse Embryo" (NCB-RS58009A). It has now been

seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology. Please do not hesitate to contact me if you have any questions.

Sincerely,
Daryl

Daryl Jason Verzosa David, PhD

Senior Editor, Nature Cell Biology
Advisory Editor, npj Biological Physics and Mechanics
Nature Portfolio

Heidelberger Platz 3, 14197 Berlin, Germany
Email: daryl.david@nature.com
ORCID: https://orcid.org/0000-0002-9253-4805

Reviewer #1 (Remarks to the Author):

I thank the authors for such a thorough revision of their manuscript "Developmental Chronology of Mouse Embryo" which I now believe is ready for publication. The detailed subsampling/saturation analysis is quite interesting, particularly highlighting the structural differences in transcript coverage and detection when using sci-RNA-seq3. The refined analyses related to transcription factor prediction, epithelial expansion and in situ validation of Nephron Progenitor Cell dynamics are also all very valuable, and I appreciate the more detailed comparison to Qui et al, 2024 in terms of coverage and performance. This is a very valuable and impressive resource.

Reviewer #2 (Remarks to the Author):

We appreciate the authors' substantial efforts in the revision. The manuscript is materially improved and now includes additional experimental and bioinformatic validation that strengthens the central observations. We have no further concerns regarding the manuscript.

Version 2:

Decision Letter:

Dear Dr Pei,

I am pleased to inform you that your manuscript, "Developmental Chronology of Mouse Embryo from 2-cell stage through birth", has now been accepted for publication in Nature Cell Biology.

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Cell Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

If you have any questions about our publishing options, costs, Open Access requirements, or our legal forms, please contact ASJournals@springernature.com

Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details. An online order form for reprints of your paper is available at <a href="https://www.nature.com/reprints/author-

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Please feel free to contact us if you have any questions.

With kind regards,
Daryl

Daryl Jason Verzosa David, PhD

Senior Editor, Nature Cell Biology
Advisory Editor, npj Biological Physics and Mechanics
Nature Portfolio

Heidelberger Platz 3, 14197 Berlin, Germany
Email: daryl.david@nature.com
ORCID: https://orcid.org/0000-0002-9253-4805

We sincerely thank the editor and all the reviewers for their time, effort, and insightful comments. Their constructive suggestions are greatly appreciated and have substantially improved the quality and clarity of our manuscript.

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

Cao, Lin, Feng, Chen, Wu et al.'s manuscript titled "Developmental Chronology of Mouse Embryo" builds a comprehensive single cell atlas of mouse embryology from the ~2 cell stage through to birth. By unifying their data collection by assay (the 10x Chromium NextGEM v3 3' UTR), they have built a robust reference comprised of ~2.2 million cells using a methodology that generally provides higher per cell transcript recovery and minimal batch artifacts, two features that can hamper high resolution analysis of differential gene expression, lineage trajectory, or cell type annotation. They then utilize this resource to highlight a number of compelling features of embryogenesis, including the comparatively delayed differentiation of epithelial subtypes (predominantly within the endoderm) as well as the ability to identify and test master regulators of key lineages (the pituitary gland TF Tub, conditional knockout of which leads to diminished gonadotrope abundance and obesity).

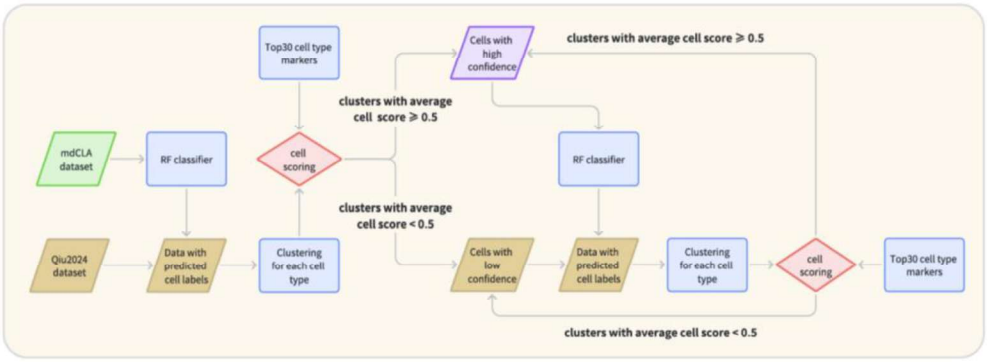
Reply: We feel sincerely grateful for you to summarize the advantages of our dataset and main findings of our manuscript. We greatly appreciate your insightful and constructive suggestions, which have significantly helped us to improve the quality and clarity of the manuscript.

Overall, the authors are correct in highlighting some of the current limitations in developmental atlas building, which in the absence of a pure assay lock and consistent techniques can lead to integration artifacts that limit their utility. Similarly, per cell transcript recovery can diminish one's ability to identify rare subtypes or to distinguish gene regulatory patterns associated with differentiation. As a resource, the data collected by the Center for Cell Lineage and Atlas group has a compelling case to make and I do not doubt it would be utilized by the field. However, given the lack of technological or analytical novelty within this manuscript, I think the authors could do more to demonstrate the value of their resource in comparison to other approaches, as well as to clarify the actual improvements that they have made. At the moment, many of the reported improvements are "showcased" (eg. by showing markers or clustering with their data versus published data slices) rather than rigorously analyzed in a unified setting. I think this does a disservice to the resource and the field overall, as it is often hard to tell how many of the reported benefits stem from comparing a custom data set to a public one, rather than reanalyzing that repository for the purposes of a fair comparison.

In particular, to me it is somewhat difficult to assess from the methods how actively versus passively the authors compared their dataset to that of the Shendure group (Qiu et al., 2024, reference 10), which could bias the interpretation that the data is itself better for these purposes. In general, I ask that the authors work to unify both data sets for the purposes of their analysis and argument. For example, if the authors claim that the improved transcript recovery is responsible for their ability to

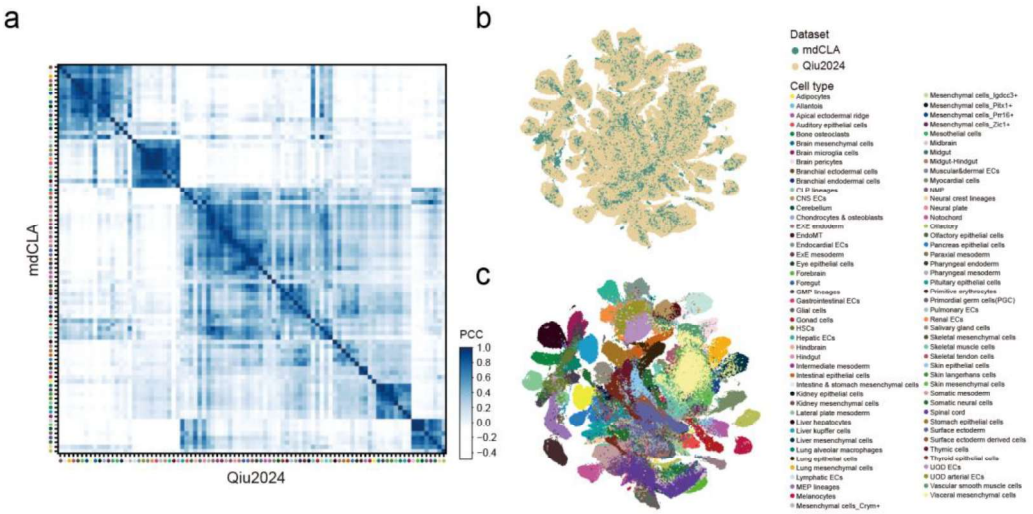
more precisely define cell types, they should process and analyze the Qiu data in the same manner: meaning, perform de novo clustering on equivalent subsections of the data (portions of the data that represent equivalent subpopulations), and then demonstrate the improved precision of their data set in identifying novel clusters (cell types) in comparison to the Qiu et al. data set using the same or similar parameters. Alternatively, the authors could elect to integrate the Qiu et al data to their own reference, which would allow them quantitatively showcase the relative expression of marker genes between the two data sets in a unified manner (meaning, they can show the cluster-level expression of variable or marker genes for every cluster between the two data sets using the same labels). The methods state that the authors have downloaded and revisualized the Qiu data using Scanpy, so either option should not be too challenging to implement and include as part of the extended data.

Reply: We apologize for the insufficient comparative analyses in the initial submission, which may have limited the evaluation of our dataset. We recognized that rigorous and unbiased comparisons are essential for the accurate interpretation and appropriate use of the atlas. In response to the reviewer's valuable suggestions, we harmonized the annotation by mapping the organ/tissue cell labels (level-2 annotation) of mdCLA to Qiu2024, based on the consideration that both atlases have sampled all organs/tissues from the intact embryos and thus share consistent organ/tissue labels.
The detailed annotation pipeline is described below (Response to Reviewer #1 Figure 1).
Step 1: A random forest (RF) classifier was trained on the mdCLA dataset.
Step 2: Predict the cell labels of Qiu2024 dataset using the RF classifier.
Step 3: Cell clustering was performed within each predicted cell label in the Qiu2024 dataset.
Step 4: For each predicted organ/tissue in Qiu2024, cells were scored by averaging marker gene expression of the top 30 organ/tissue-specifc marker genes derived from mdCLA. Then an average score was calculated for each cluster. Clusters with an average score ≥ 0.5 were defined as high-confidence clusters, whereas those with scores < 0.5 were designated as low-confidence cells.
Step 5: A new RF classifier was trained using the high-confidence cluster cells of Qiu2024, and the top 30 marker genes for each cell label in the Qiu2024 dataset were identified.
Step 6: Predict the cell labels with low-confidence cells of Qiu2024 using the new RF classifier.
Step 7: Cell clustering was performed within each newly predicted cell label.
Step 8: For each predicted organ/tissue in Qiu2024, cells were rescored using the top 30 marker genes derived from Qiu2024, and average cluster scores were calculated. Clusters with average scores ≥ 0.5 were classified as high-confidence, whereas the remaining clusters were designated as low-confidence.
Step 9: The labels of the remaining low-confidence cells were assigned as the nearest high-confidence cells in PCA space.
Final outcome: using this annotation strategy, a total of 11,441,407 cells in the Qiu2024 dataset were assigned 89 unified organ/tissue labels. Then we performed all comparative analyses of two datasets with the unified labels. The comparative analysis results have also been incorporated into Fig 2, Extended Data Figs 3 − 4, and lines 128 − 231 of the revised manuscript.



Response to Reviewer #1 Figure 1. Workflow diagram for cross-dataset cell label prediction

For visualization, we integrated the two datasets using Harmony algorithm. We found cells with the same labels clustered together and showed very similar gene expression profiles. These results validated the high accuracy of cell label transfer in the Qiu2024 dataset (Response to Reviewer #1 Figure 2a-c).



Response to Reviewer #1 Figure 2. Integration of two atlases. a. Pearson correlation coefficient (PCC) heatmap of 89 unified cell labels between two datasets, where blue indicates high correlation and white indicates low correlation. The mean values of the Harmony-corrected principal component analysis (PCA) of each cell type were used for calculation. b-c. UMAP visualization of the integrated result, colored by datasets (b) and organ/tissue labels (c).

Under the unified cell type annotation framework, we systematically evaluated how the two key metrics of single cell atlases, **cell coverage** and **transcript coverage**, contribute to the cell type identification and the gene regulatory network (GRN) inference. This analysis reveals the intrinsic limitations of each atlas and underscore the complementary value of our resource, as outlined below:

1) Both high cell coverage and high transcript coverage facilitate the identification of cell subtypes in single-cell atlases.
2) Increasing the number of cells improves the number of detected genes in pseudo-bulk profiles.
3) However, increased cell number alone is insufficient to recapitulate the gene expression

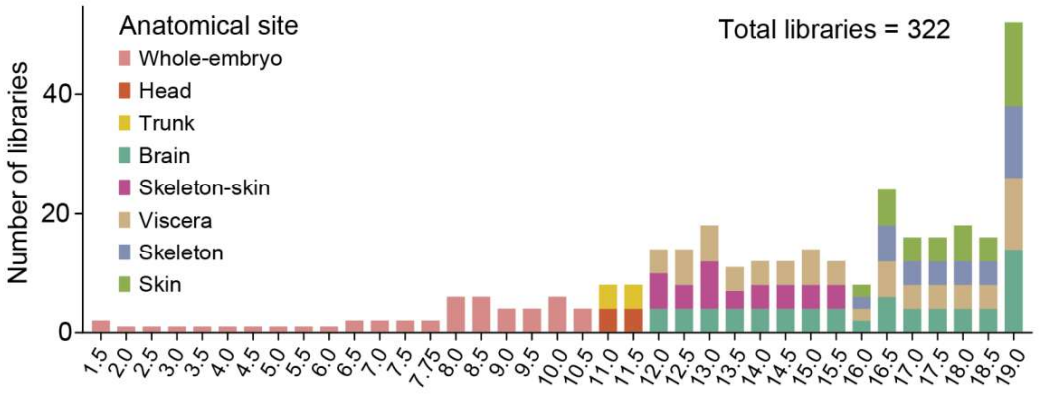
patterns obtained by deep-sequencing approaches.

4) Deep-sequencing datasets provide distinct advantages for transcriptional gene regulatory network (GRN) discovery.
5) Integrative analysis of the two atlases could mitigate their respective limitations and improve data-driven biological discovery.

From the same point of view, I think it is equally important that the authors explicitly show how their decision to dissect and sample distinct tissue compartments may bias their data set versus Qiu et al, which I believe sampled nuclei from entire embryos without dissection or tissue specific digestion/processing. The authors should use this prior data set to state at what resolution their work can make accurate/unbiased statements about cell state composition within a developing embryo, for example by comparing matched time points and/or individual tissue systems to the Shendure group data, and then calculating the relative divergence (distance) between them. It may be possible that the Shendure data set and this data set diverge at the level of the full embryo, but that they are more comparable within specific subcompartments (epithelium, brain, or other). These types of analyses would be best conducted on unified cell state labels as requested above.

Reply: We appreciate this insightful comment regarding the potential biases introduced by tissue-specific dissection and sampling. We agree that both whole-embryo nuclear extraction (as performed by Qiu et al.) and our tissue-specific single-cell dissociation introduce distinct technical biases; specifically, different cell types respond variably to enzymatic digestion versus mechanical disruption, and the RNA profiles captured from whole nuclei versus whole cells are inherently different.

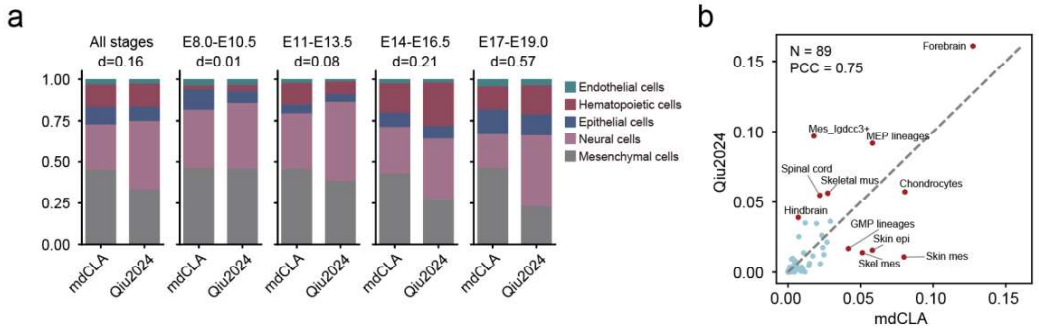
To address the reviewer's concern regarding sampling transparency, we have added a new figure and supplementary table (Response Fig. 3; Supplementary Table 1) summarizing the sequencing libraries for each tissue compartment across developmental stages. Our protocol utilized tissue-specific dissociation methods tailored to the unique adhesion properties of different compartments to maximize cell recovery. While we aimed for balanced sampling, we have explicitly noted that E16.0–E19.0 skin tissue was processed separately, resulting in a higher relative cell count for this compartment. We have updated the manuscript to discuss how these sampling decisions impact the resolution of our cell state composition analyses in comparison to whole-embryo approaches (Response to Reviewer #1, Fig. 3 and Supplementary Table1).



Response to Reviewer #1 Figure 3. Barplot showing the number of single cell sequencing libraries per stage, color indicates the anatomical sites of the libraries.

We also agree that assessing potential biases introduced by our tissue-specific dissection strategy is essential for a rigorous comparison with whole-embryo sampling methods, such as those employed by Qiu et al.. To this end, we grouped developmental stages (E8.0–E19.0) into four intervals encompassing all sampled stages and compared the relative proportions of 5 major cell lineages (epithelial, mesenchymal, endothelial, neural, and hematopoietic cells) between the two datasets (Response to Reviewer #1, Fig. 4a). Our analysis reveals that the overall, cell composition was largely consistent over developmental timepoints. We noted that the Qiu et al. dataset captured a slightly higher proportion of neural cells during late development; this likely reflects the inherent advantage of single-nucleus sequencing in profiling mature neurons, which are often underrepresented in droplet-based single-cell protocols due to their size and fragility.

We then compared the cellular composition of unified organ/tissue labels. The results showed that most tissues had similar cellular distributions (87.6%, 78/89), with only minor differences in 12.4% of tissues (11/89) (Response to Reviewer #1, Fig. 4b). Specifically, Qiu2024 contained higher proportion of the brain, spinal cord, and skeletal muscle cells, potentially due to the prefential capture of multinucleated cells or neurons. In contrast, our mdCLA dataset exihibited a higher representation of skin cells. We therefore emphasize that sequencing platforms and sampling strategies differences can lead to variations in cellular composition. We have clarified these points in the revised manuscript to assist readers in interpreting the resolution and composition of our data.



Response to Reviewer #1 Figure 4. a. Stack barplot showing the cell composition of 5 cell groups in different stages. We also calculated the Pearson distance (d=1-PCC) of cell proportions between two datasets. Distance increases with developmental timepoints. b. Scatter plot shows the cell

composition of 89 major cell types between two datasets.

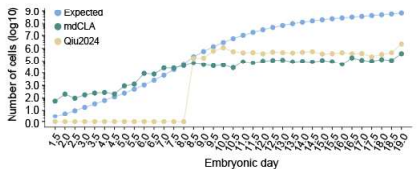
Similarly, the key benefit of combinatorial indexing (the Shendure group's method) is that it can sample a larger number of cells per embryo at a reduced per cell transcript count. Here, I would like to see the authors include as extended data comparable estimates of the per embryo "coverage" between the two groups (the fraction of cells sequenced per embryo as a function of developmental time), even if they must proceed from published estimates of the number of cells per embryo stage. I am particularly surprised at how visually similar the cell coverage appears to be when the authors compare the two atlases for different compartments (for example in Figure 2 as well as ED Figures 3 and 4), particularly given the larger number of cells that Qiu et al sampled overall. As a minor comment, the authors should include n's on these plots to highlight the number of cells included, and be more explicit about which timepoints these cells are derived from in both data sets.

Reply: We thank the reviewer for the constructive and detailed comments. To address the question of per-embryo coverage, we estimated the total expected cell counts across mouse developmental stages and compared these to the number of cells captured in our study (mdCLA) and Qiu2024. We applied polynomial regression (degree=3) to fit the curve between embryonic day and log2(cell number) and estimated the expected cell numbers of intact embryos across developmental stages. Specifically, for E8.5–E19.0, we used the formula provided by Qiu2024 directly (PMID: 38355799). For earlier stages (E1.5–E8.0), we constructed the formula based on experimentally reported cell numbers (PMID: 10529424, PMID: 37471320) (Response to Reviewer #1, Fig. 5a, b and Supplementary Table 3; see Methods). Cell coverage was calculated as the ratio of sequenced cell number to the expected total cell number per stage. Our analysis shows that mdCLA provides high coverage for stages prior to E8.0, these timepoints not covered by Qiu et al. Between E8.5 and E19.0, cell coverage in Qiu2024 was approximately six fold higher than in mdCLA (Response to Reviewer #1, Fig. 5c).

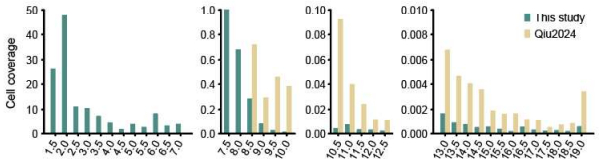
a

Merge time (E)	mdCLA day (E)	Qiu2024 day (E)	mdCLA cell numbers	Qiu2024 cell numbers	Expected cell number	Estimation formula (cell numbers ~ day)
1.5	1.5	0	52	0	2	
2	2	0	190	0	4	
2.5	2.5	0	89	0	8	
3	3	0	167	0	16	
3.5	3.5	0	231	0	31	
4	4	0	259	0	59	
4.5	4.5	0	197	0	114	
5	5	0	865	0	226	
5.5	5.5	0	1219	0	468	$\log_2(\text{cell numbers}) = 0.027661\times day^3-0.296459\times day^2+2.923354\times day-2.843395$
6	6	0	8605	0	1023	
6.5	6.5	0	7596	0	2401	
7	7	0	23552	0	6128	
7.5	7.5	0	23367	0	17263	
8	7.75,8.0	0	36971	0	54444	
8.5	8.5	8.25	60819	153597	213382	
9	9	9.0,8.75	45190	164302	561462	
9.5	9.5	9.5,9.25	36238	613423	1342163	
10	10	9.75,10.0	41143	1123262	2932104	
10.5	10.5	10.5,10.25	25151	541356	5888572	
11	11	10.75,11.0	81035	436495	10936130	
11.5	11.5	11.5,11.25	63784	455968	18893277	
12	12	11.75,12.0	94601	368115	30542716	
12.5	12.5	12.25,12.5	105739	499629	46476300	
13	13	12.75,13.0	112374	454123	66964679	
13.5	13.5	13.5,13.25	81476	426489	91900357	$\log_2(\text{cell numbers}) = 0.011369\times day^3-0.583861\times day^2+10.397036\times day-35.469755$
14	14	13.75,14.0	90319	490303	120840515	
14.5	14.5	14.333,14.25	78568	547693	153143551	
15	15	14.75,15.0	105388	358165	188166557	
15.5	15.5	15.25,15.5	83077	371932	225481312	
16	16	15.75,16.0	49385	445164	265075667	
16.5	16.5	16.5,16.25	169238	372215	307529779	
17	17	16.75,17.0	108325	386649	354184295	
17.5	17.5	17.25,17.5	90392	213288	407345612	
18	18	17.75,18.0	124404	333404	470603562	
18.5	18.5	18.25,18.5	105650	458152	549379705	
19	19	18.75,19.0	375244	2227683	651901611	

b



c



Response to Reviewer #1 Figure 5. a.Table of the expected, cell number per developmental stage of mdCLA and Qiu2024. b. Dot and line plot showing the expected, mdCLA sequencing, Qiu2024 sequencing cell number per developmental stage. b. Barplot showing the cell coverage of each atlas per developmental stage.

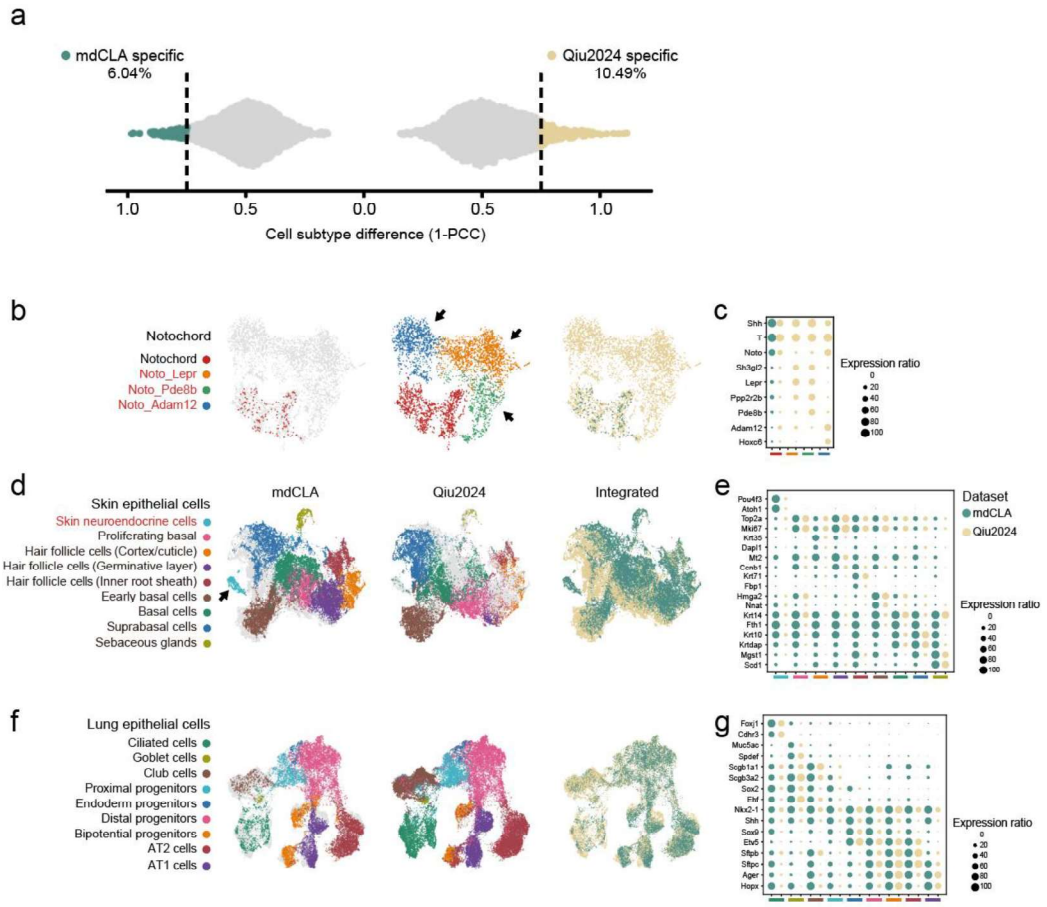
To investigate how cell coverage versus transcript coverage (sequencing depth) influences cell-type discovery, we implemented a unified clustering and annotation framework. We applied an identical pipeline to both datasets and perform cell clustering: identification of top HVGs using scanpy (min_disp=0.5, min_mean=0.0125, max_mean=3), PCA analysis (top 50 PCs), searching for k-nearest neighbor cells (n_neighbors=10) and Leiden clustering (n-neighbors=15, resolutiom=0.3). Clusters were defined as dataset-specific if their maximum Pearson correlation coefficient (PCC) to any cluster in the reference dataset was less than 0.25; otherwise, they were considered shared.

Using this framework, we identified that 10.49% (116/1106) of cell types in Qiu2024 and 6.04% (74/1225) in mdCLA are dataset-specific (Response to Reviewer #1, Fig. 6a). For example, three notochord cell types were identified as Qiu2024-specific (Response to Reviewer #1, Fig. 6b, c). Our results demonstrate two complementary strengths:

1) High cell coverage (Qiu2024) facilitates the detection of particular cell populations. For instance, three specific notochord cell types were uniquely identified in Qiu2024 due to the high cell number captured.

2) High transcript coverage (mdCLA) facilitates the identification of cell types defined by low-abundance transcripts. For example, skin neuroendocrine cells were identified in mdCLA but not in Qiu2024. While these cells likely exist in the Qiu2024 dataset, the expression of canonical markers (e.g., *Pou4f3, Atoh1, Krt20*) was insufficient for robust clustering and identification (Response to Reviewer #1, Fig. 6d, e and Fig. 2c).

We apologize for an incorrect conclusion in the original manuscript (Fig. 2c–d), where we stated that pulmonary ciliated cells were unique to mdCLA. Under the unified annotation framework, we demonstrated that Qiu2024 does contain pulmonary ciliated cells (Nkx2-1+/Sox2+/Foxj1+), which were misannotated as ependymal cells in the original Qiu2024 annotations (Response to Reviewer #1, Fig. 6f–g). Both pulmonary ciliated cells and ependymal cells express Foxj1; however, pulmonary ciliated cells additionally express the lung-specific marker Nkx2-1, whereas ependymal cells specifically express Uchl1 (PMID: 38155158). We have removed this incorrect example from the manuscript and updated our comparative analysis accordingly. We thank the reviewer for drawing attention to this issue, which allowed us to correct this error.



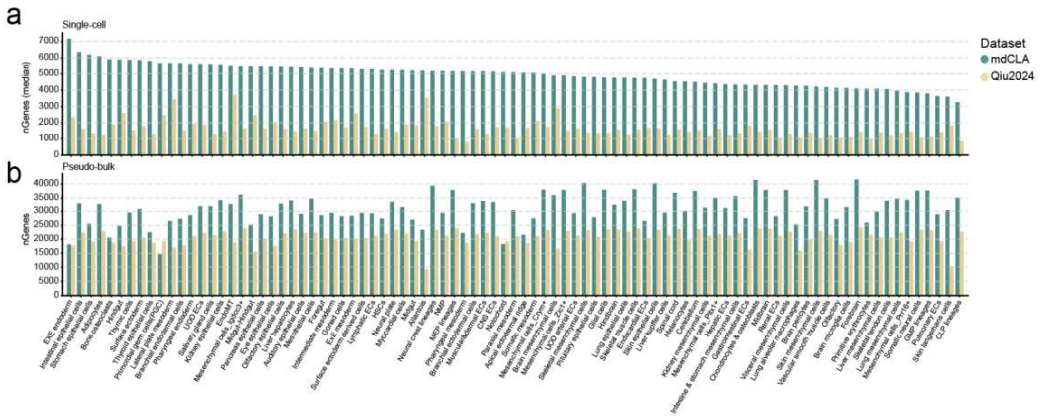
Response to Reviewer #1 Figure 6. a. Scatter plot showing the dataset-specific cell type. Each dot represents a cell type and green color indicates the Qiu2024 specific, while yellow color indicates the Qiu2024 specifc. b-g. The integration results (b, d, f) and the marker gene expression (c, e, g) of notochord, skin epithelial cells and lung epithelial cells from two datasets. The arrow points to a dataset-specific cell type.

I am also curious about the author’ s explanation that the larger number of DEGs reflects higher per cell transcriptome coverage and ask for more analysis to this point. The combinatorial indexing strategies utilized by Qiu et al al sample much larger cell numbers (~11M to 2M cells, a roughly 5-fold difference) with only an ~2-3 fold decrease in the number of recovered transcripts per cell. In general, my expectation would be that lower per cell transcript sampling would be compensated by

higher numbers of sampled cells, but this doesn't appear to be true in the data presented in Figure 2 or Extended Data Figures 3 and 4, which show striking disparities in the expression of the same marker genes. These results suggest that no amount of additional sampling would adequately recover an accurate "pseudobulk" based assessment of a given cell state's transcriptome. Here, I would ask that the authors demonstrate the degree to which this may or may not be true by showing the relative correlation between normalized pseudobulk-level transcript counts between the two data sets on unified labels, both for the total transcriptome and for marker genes, and to test in certain cases whether or not this correlation is stable as a function of sampled cells.

Reply: We thank the reviewer for highlighting this important issue regarding the relationship between per-cell transcript coverage and total cell sampling. To assess whether lower per-cell transcript coverage can be compensated by increased cell numbers, we performed a series of downsampling and correlation analyses.

First, we generated normalized pseudobulk expression profiles for each unified cell label, scaled to 10,000 total counts. While pseudobulk aggregation did reduce the disparity in gene detection (nGenes), it did not eliminate it. Specifically, the ~4-fold difference in nGenes at the single-cell level remained a ~1.2-fold difference at the pseudobulk level, suggesting that certain transcripts remain undetected in the Qiu2024 dataset regardless of aggregation (Response to Reviewer #1, Fig. 7a–b).



Response to Reviewer #1 Figure 7. a. Barplot showing the number of median detected genes (expression > 0) of cells of each organ/tissue. b. Barplot showing the number of detected genes (expression > 0) of each pseudobulk.

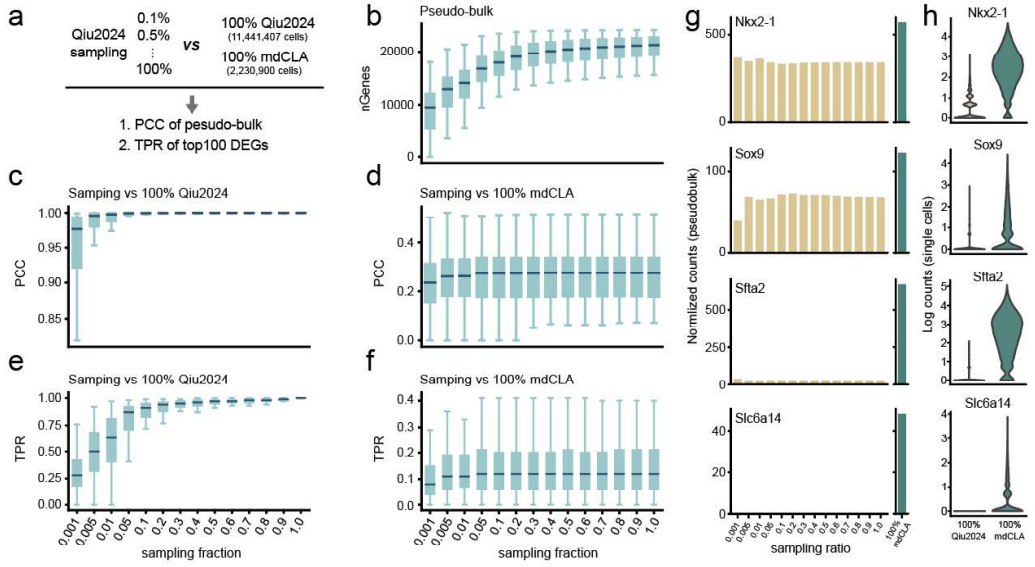
We next investigated to what extent the cell number influences gene detection. We downsampled the Qiu2024 dataset across a gradient of fractions (0.1%, 0.5%, 1%, 5%, 10%, …, 90%, and 100% of the total 11,441,407 cells) and calculated the pseudobulk of unified cell labels (Response to Reviewer #1, Fig. 8a). Our results demonstrate that while increasing cell numbers initially improves gene detection and Pearson correlation coefficients (PCC), this benefit reaches a plateau once sampling exceeds ~20% of the total population (Response to Reviewer #1, Fig. 8b).

Correlation analysis between downsampled and 100% pseudobulk of Qiu2024 exhibited a similar trend: the Pearson correlation coefficient (PCC) rise rapidly with increasing cell numbers at low sampling fractions (<5% of total cells) but plateaus at higher sampling fractions (>5% of total cells)

(Response to Reviewer #1, Fig. 8c). Furthermore, increased cell numbers in Qiu2024 were insufficient to recapitulate the gene expression patterns observed in mdCLA, with a max median PCC of 0.26 (Response to Reviewer #1, Fig. 8d), indicating that even massive cell numbers cannot fully recapitulate the expression patterns captured by the higher-depth mdCLA dataset.

This "saturation effect" is particularly evident in marker gene analysis. We calculated the top100 organ/tissue specific marker genes (ranked by $\log_2$ fold change) from 100% mdCLA and Qiu2024 datasets, respectively. We adopted these marker genes as the gold standard and calculated the true positive rate (TPR) of top100 marker genes identified by downsampled Qiu2024 datasets. Similarly, TPR improved significantly with increased cell numbers at low sampling fractions (<20% of total cells) but showed minimal improvement at higher fractions (>20% of total cells) (Response to Reviewer #1 Figure 8e-f).

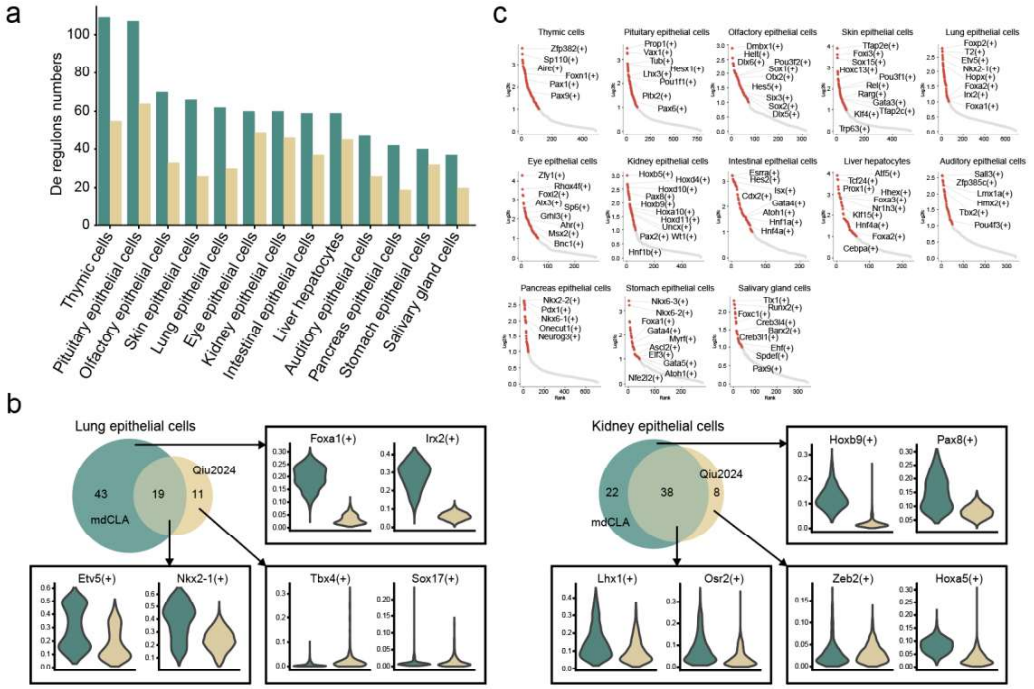
Taking lung epithelial cells as a case study. We analyzed the expression of four well-established developmental markers: *Nkx2-1* (master transcription factor of all lung epithelial cells), *Sox9* (distal alveolar progenitor marker), *Sfta2* (surfactant associated 2) and *Slc6a14* (solute carrier family 6). The pseudobulk expression level of *Sox9* increased with cell numbers at low sampling fractions but reached a plateau above 5% of total cells. Even at 100% cells, its expression level in Qiu2024 did not reach that observed in mdCLA. Notably, the expression of *Nkx2-1*, *Sfta2* and *Slc6a14* was not associated with the increase in cell number (Response to Reviewer #1 Figure 8g). Among them, *Sfta2* and *Slc6a14* were nearly undetectable in Qiu2024 at single cell level (Response to Reviewer #1 Figure 8h). Our analysis suggests that increasing cell numbers provides limited enhancement in marker gene detection for shallow-depth sequencing data.



Response to Reviewer #1 Figure 8. a. Dataset downsampling and testing strategy. b. Number of detected genes (expression > 0) of pseudobulk data for 89 unified cell labels across 14 sampling factions. c. PCC of downsampled Qiu2024 datasets with 100% Qiu2024 dataset. d. PCC of downsampling Qiu2024 datasets with 100% mdCLA dataset. e-f. True positive rate (TPR) of DEGs identification across downsampling fractions. Qiu2024 top100 DEGs as gold standard (e), mdCLA top100 DEGs as gold standard (f). g. Barplot showing the pseudobulk expression of four well-

established lung epithelial cell marker genes. Yellow bar indicates the Qiu2024 datasets, green bar indicates the 100% mdCLA dataset. (h) Violin plot showing the marker gene expression at single cell level.

We hypothesize that the deep transcript coverage could facilitate the identification of transcriptional factor co-expression modules (TF modules) (see Methods in revised manuscript). By comparing the epithelial tissue specific TF modules identified by two atlases respectively, we showed that mdCLA yielded more differentially expressed TF modules than Qiu2024 (t-test, p-value < 0.01) (Response to Reviewer #1 Figure 9a and Supplementary Table 4). For instance, in epithelial cells derived from the lung and kidney, the TF module scores of mdCLA were significantly higher than those of Qiu2024 (Response to Reviewer #1 Figure 9b). We present 13 tissue-specifically expressed TF modules identified by mdCLA, which may serve as a reference to further exploring their functional roles during developmental processes (Response to Reviewer #1 Figure 9c).



Response to Reviewer #1 Figure 9. a. Bar plot showing the number of differentially expressed TF co-expressed modules identified by two datasets, respectively. b. Venn plot showing the intersection of organ-specific TF modules identified by each dataset. Violin plot shows the TF module score between two datasets, calculated by AUCell. c. Rank plot showing the top TF modules of 13 epithelial organs or tissues identified by mdCLA. Module scores were calculated by AUCell algorithm, differentially expressed modules were identified by t-test, with thresholds set as adjusted p-value < 0.001 and $\log_2$ fold change > 1.

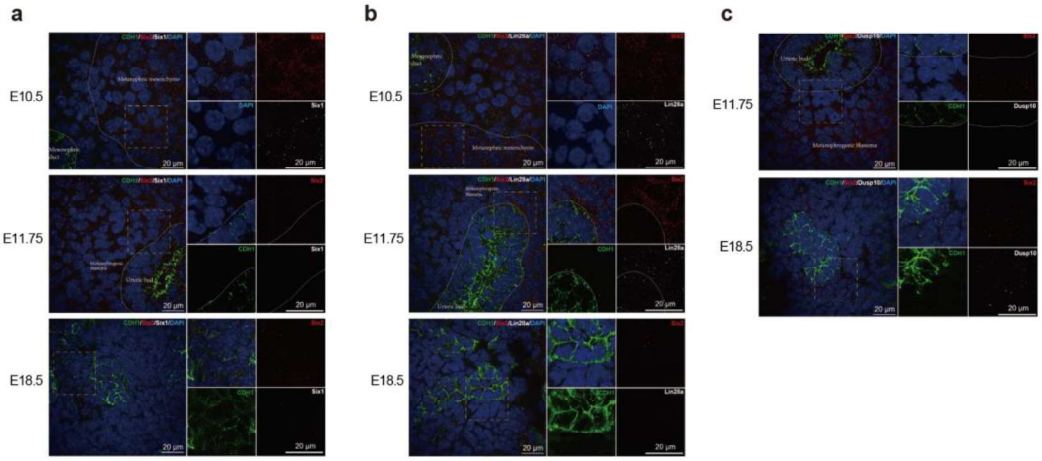
In summary, while lower per-cell transcript coverage can be partially compensated by increasing cell numbers, this effect is bound. In the case of the Qiu2024 dataset, the enhancement saturated above 2,000,000 cells (20% of total cells). In addition, our results demonstrated that gains in gene expression level saturate well before reaching the full cell count (at 5% of total cells). These findings support our conclusion that high per-cell sequencing depth is indispensable for the high-

fidelity detection of marker genes and regulatory modules.

Finally, although this manuscript is being considered as a Resource, it is worth highlighting that two novel descriptive findings using this data (the terminal differentiation of metanephric progenitors or the synchronized late timing of epithelial/endodermal differentiation) are not validated with additional in situ data, such as in situ hybridization or immunofluorescence. While this may not be a major requirement for a Resource article, the authors do state that their methodology allows them to discover these previously unobserved processes, so some additional validation that these markers are present in the tissues with the dynamics described seems worth considering for inclusion in a revised manuscript.

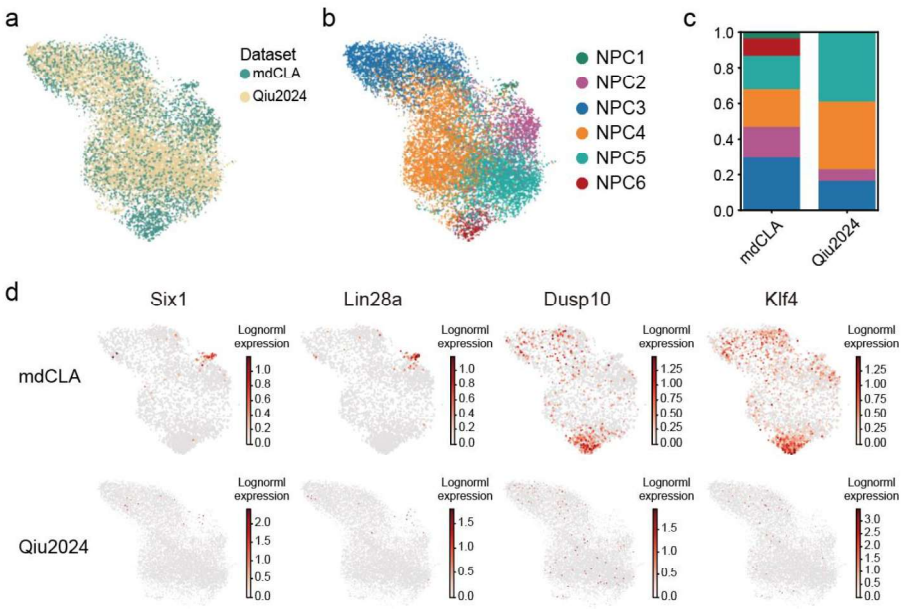
Reply: We thank the reviewer for the valuable suggestions. We have addressed these concerns through additional experimental validation and computational analysis:

1) Validation of nephron progenitor dynamics: Our single-cell analyses identified two distinct nephron progenitor populations: **NPC1** (characterized by *Six1* and *Lin28a*), which diminishes by E11.0, and **NPC6** (enriched for immune-related genes like *Dusp10*), which emerges after E16.0 (Fig. 3b-f of revised manuscript). To validate these findings, we performed single-molecule fluorescence in situ hybridization (smFISH) to track the expression of these markers within the *Six2*$^{+}$ population at E10.5, E11.75, and E18.0. These results confirm the temporal dynamics observed in our sequencing data (Response to Reviewer #1, Fig. 10a – d).



Response to Reviewer #1 Figure 10. a-b. smFISH validation of NPC1 marker genes. c-d. smFISH validation of NPC6 marker genes.

We also showed that neither the NPC1 nor the NPC6 populations, nor their associated marker genes, were detected in the Qiu2024 dataset.



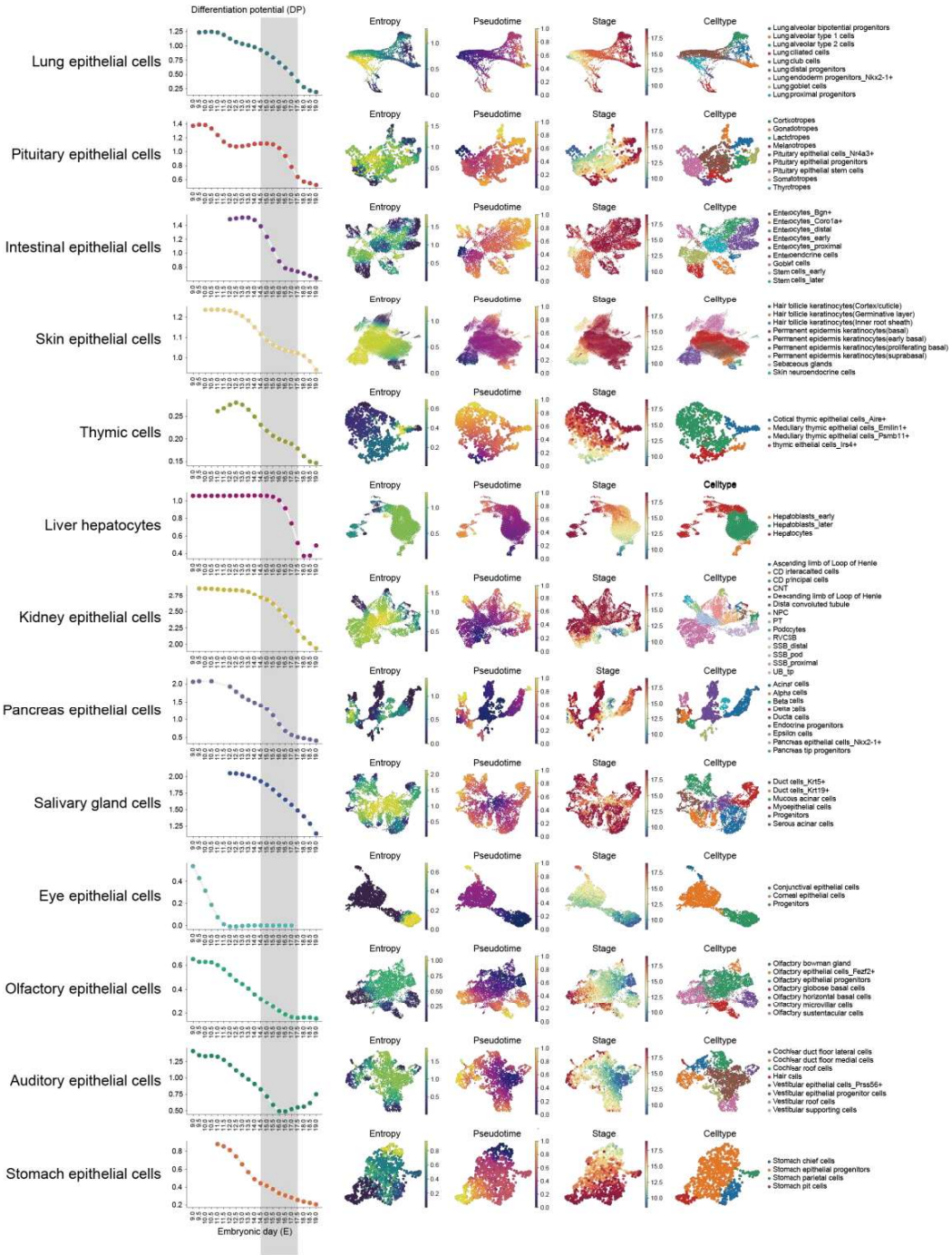
Response to Reviewer #1 Figure 11. a-b. Harmony integration of mdCLA and Qiu2024 NPC populations, colored by dataset (a) and cell subtype (b). c. Stack barplot showing the NPC proportion between two datasets. d. UMAP showing the marker gene of NPC1 (Six1 and Lin28a) and NPC6 (Dusp10 and Klf4) in mdCLA (upper panel) and Qiu2024 (bottom panel).

2) Regarding the accelerated epithelial differentiation: The rapid differentiation of endodermal- and ectodermal-derived lineages between E14.5 and E18.5 is consistent with established knowledge. Previous studies have shown that during embryonic day (E)14.5 to E18.5, endodermal- and ectodermal-derived epithelial cells undergo rapid differentiation into lineage-committed cell types across multiple organs, such as the intestine (PMID: 21978911) and pituitary (PMID: 32163208). Prior to E14.5, the murine fetal intestine primarily elongates and expands, forming a pseudostratified epithelium composed of proliferative progenitor cells. Between E14.5 and E16.5, non-proliferative villus regions and proliferative intervillous regions emerge, and these immature domains subsequently give rise to most differentiated intestinal epithelial cell types. Similarly, in the developing pituitary gland, differentiated melanotroph, somatotroph, lactotroph, and gonadotroph lineages first appear during E14.5 – E18.5. We have incorporated these studies as references to support the interpretation of our findings.

3)

4) In addition, we also employed Palantir algorithm to validate the phenomenon of accelerated differentiation. Palantir quantifies the probability of each cell differentiating into distinct cell fate branches, thereby enabling the calculation of entropy over branches as cellular differentiation potential (DP). Undifferentiated cells exhibit high DP owing to their multiple differentiation potentials, whereas DP declines as cell fates become progressively specified. We applied Palantir to analyze the DP of 13 epithelial cell types and their

temporal dynamics. The results revealed a rapid decline in DP across the most epithelial cell types between E14.5 and E17.5, indicating that high rate of fate branching events occur during this period (Response to Reviewer #1, Fig. 12).



Response to Reviewer #1 Figure 12. Trajectory analysis of 13 epithelial cell types using Palantir. From left to right, the average differentiation potential (DP) of cells at each developmental stage; UMAP displays the cells' DP (entropy), pseudotime, developmental stage and cell type.

Reviewer #2 (Remarks to the Author):

This study presents a comprehensive single-cell transcriptomic atlas that offers a detailed view of mouse embryonic development from E1.5 to E19.0. The key strengths of this work include: (1) exceptional data breadth and depth, covering 37 developmental time points and approximately 2 million cells; (2) high data quality in terms of gene detection and cell-type annotation, outperforming existing atlases (e.g., Qiu et al., 2024); and (3) identification of two distinct nephron progenitor cell (NPC) populations with intriguing and non-overlapping temporal dynamics. The resulting mdCLA atlas represents a valuable resource for the developmental biology community. However, several aspects require further attention to enhance the impact and clarity of the study.

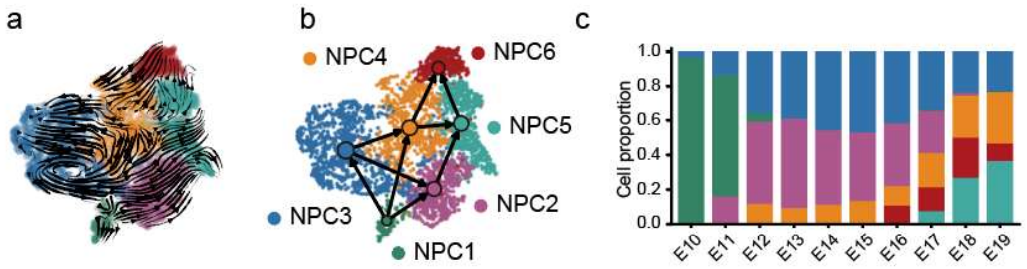
Reply: We thank the reviewer for the thoughtful summary of our main findings and for expressing interest in our work. We are grateful for the insightful comments, which have guided substantial improvements to the manuscript. As detailed below, we have provided new results and analyses that address all the reviewer's concerns.

Major Issues

1. While the dataset is rich, the analyses presented are relatively superficial and do not fully exploit its potential. For example, are NPC1 and NPC2 developmentally related? Their non-overlapping temporal windows (E13 – E15) suggest distinct origins. This relationship could be explored computationally using tools such as pseudotime analysis or RNA velocity, which may reveal lineage trajectories and clarify whether these populations arise from a common precursor or independent lineages.

Reply: We thank the reviewer for raising this point. To improve clarity and readability, we have reassigned NPC cluster numbers in the revised manuscript such that the numerical labels reflect the relative developmental timing of the NPC subpopulations. Specifically, the original NPC2 has been renamed NPC6, as it emerges only at later developmental stages. In contrast, NPC1 retains its original designation, as it represents the earliest NPC population during development.

We have analyzed the NPC trajectories using RNA velocity (Response to Reviewer #2, Fig. 1a–b). These analyses indicate that NPC1 gives rise to NPC2–NPC6 and represents the earliest-emerging subtype among all NPC populations (Response to Reviewer #2, Fig. 1c). This finding aligns with metanephric developmental processes reported in the literature (PMID: 34594045). During early metanephric development (around E10.0), the ureteric bud (UB), derived from the mesonephric duct, invades the metanephric mesenchyme (MM). Signals from the UB tip induce MM cells to adopt an NPC identity, which subsequently differentiate into nephrons. These results support the conclusion that NPC1 resides within the metanephric mesenchyme and serves as the ancestral population derives all other NPC populations.



Response to Reviewer #2 Figure 1. a-b. RNA velocity analysis of NPC populations using scVelo. PAGA trajectory paths were mapping on UMAP plot. c. Barplot showing the NPC proportion over time.

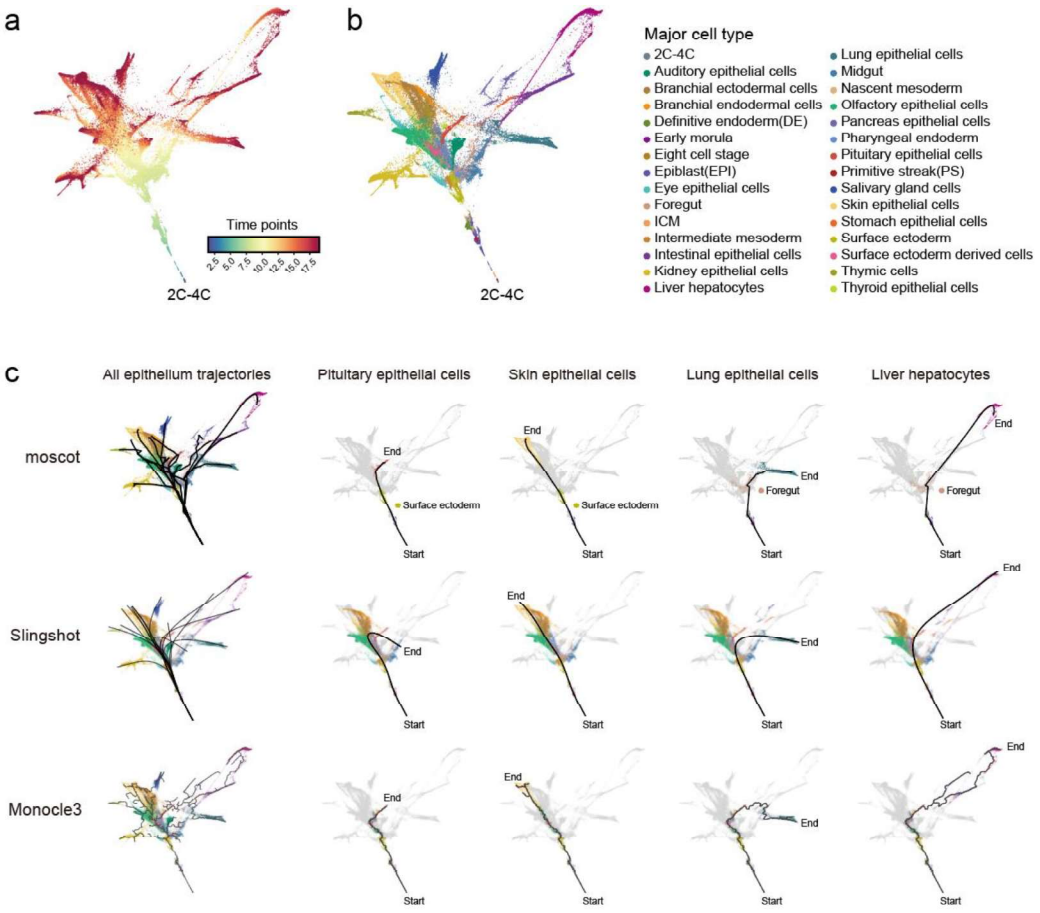
2. The concept of "accelerated differentiation" in epithelial cells (Figure 3) is intriguing but lacks robust validation. Trajectory inference using methods such as Slingshot, Monocle 3, or Palantir would provide stronger support for this claim. In particular, the optimal transport-based inference shown in Figure 3B is insufficiently explained — both the methodology and rationale should be clarified, ideally with benchmarking against more conventional trajectory tools.

We sincerely thank the reviewer for the valuable comments. The accelerated epithelial development shown in Fig. 3 of the original manuscript (now Fig. 4) reflects the observation that a larger number of newly emerging epithelial cell subtypes arise during E14.5–E17.5 compared with other developmental stages. We would like to clarify that the conclusion regarding accelerated epithelial development is independent of the optimal transport (OT) algorithm (moscot). OT was used exclusively for inferring developmental trajectories. Therefore, we validated the **developmental trajectory** and **acceleration of epithelial development** results, separately.

1) Benchmarking developmental trajectory inference

We reconstructed epithelial developmental trajectories from the E1.5 to E19.0 using two pseudotime-based methods, Slingshot and Monocle3, and compared them with trajectories inferred by OT. For visualization, trajectories from all three methods were projected onto a two-dimensional embedding (Response to Reviewer #2, Fig. 2a–c). We focused on representative epithelial lineages, including surface ectoderm-derived pituitary and skin epithelial cells, as well as endoderm-derived lung and liver epithelial cells.

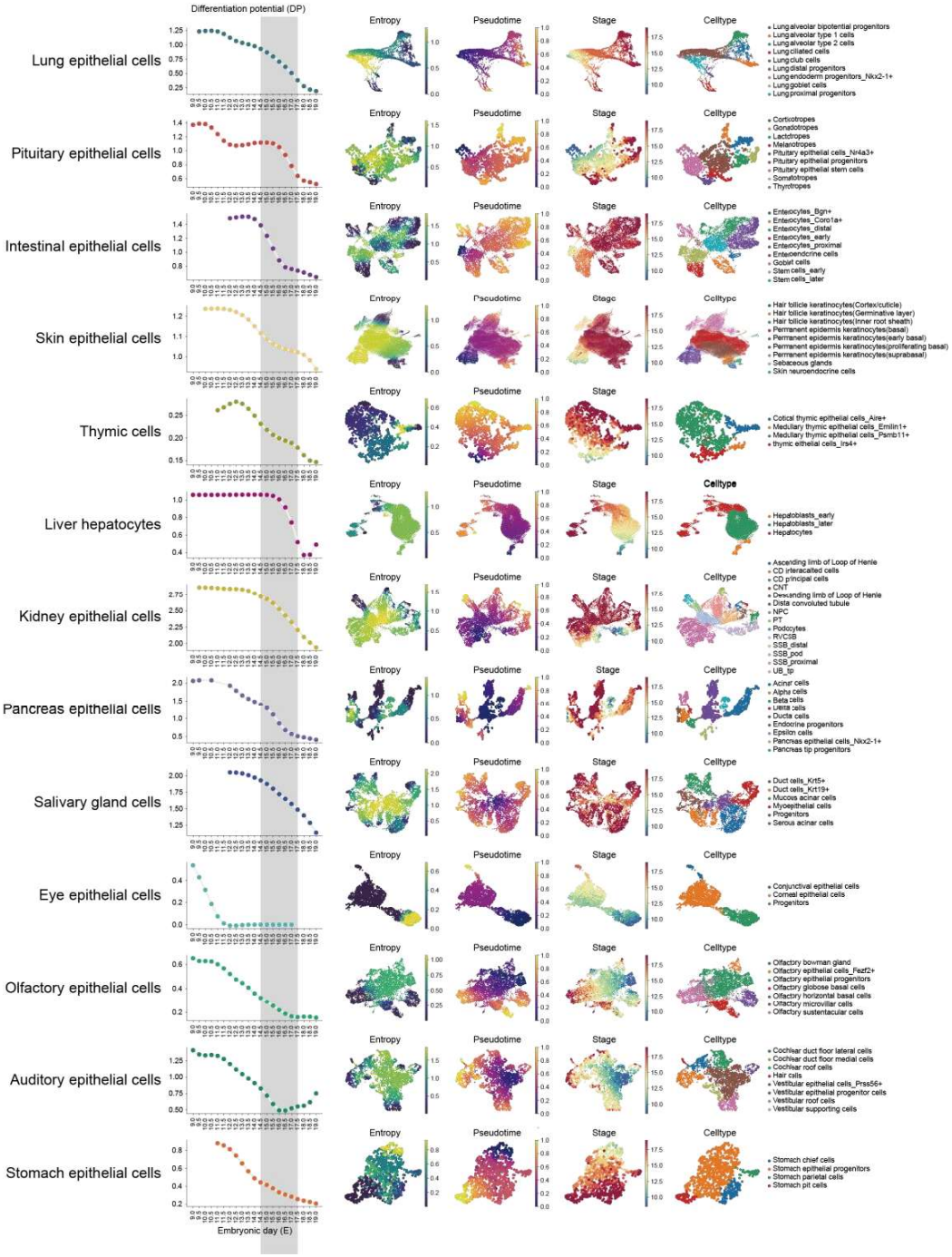
All three methods produced consistent trajectories for pituitary and skin epithelial lineages, capturing their progression from surface ectoderm to terminally differentiated states. In contrast, only OT correctly inferred the foregut (endoderm) origin of lung and liver epithelial cells; Slingshot and Monocle3 incorrectly assigned their origins to surface ectoderm. These comparisons indicate that OT is better suited for developmental datasets with dense time points, such as mdCLA. In addition, we initially chose OT for trajectory inference as it calculates differentiation trajectories from real developmental time rather than pseudotime, enabling direct visualization of lineage-specific gene expression dynamics at each developmental stage (Extended Data Fig. 9d, e in revised manuscript).



Response to Reviewer #2 Figure 2. a-b. Force-directed layout of epithelial cell development. The cells were downsampled for better layout using harmonyTS algorithm. Colored by developmental stage (a) and major cell type (b). c. Trajectory path of moscot, Slingshot and monocle3 mapped on 2D layout as (a).

2) Acceleration of epithelial development

Inspired by the reviewer's comment, we employed Palantir to quantitatively assess developmental acceleration. Palantir quantifies the probability of each cell differentiating into distinct cell fate branches, thereby enabling the calculation of entropy over branches as cellular differentiation potential (DP). Undifferentiated cells exhibit high DP owing to their multiple differentiation potentials, whereas DP declines as cell fates become progressively specified. We applied Palantir to analyze the DP of 13 epithelial cell types and their temporal dynamics. The results revealed a rapid decline in DP across the most epithelial cell types at E14.5–E17.5, indicating that high rate of fate branching events occur during this period. These results supported the conclusion of accelerated epithelial development presented in our manuscript.

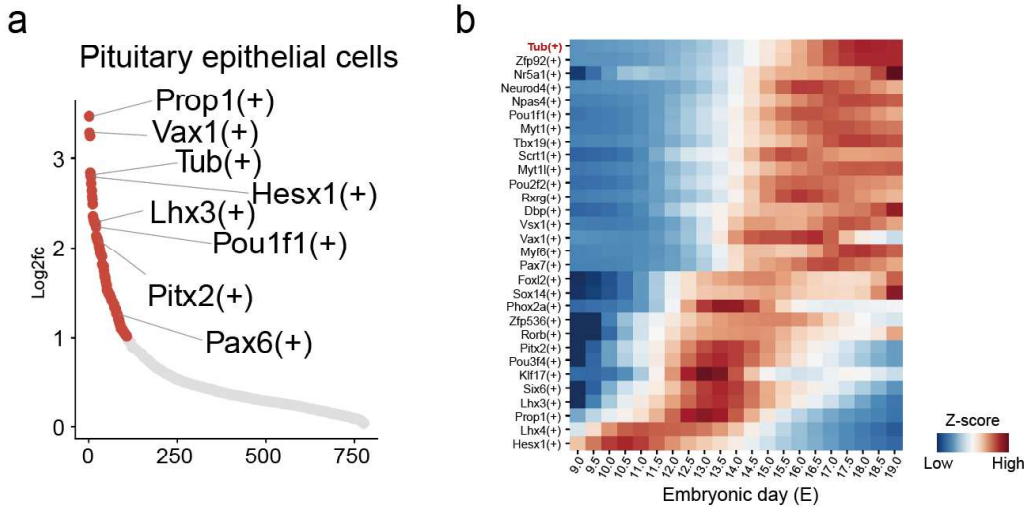


Response to Reviewer #2 Figure 3. Trajectory analysis of 13 epithelial cell types using Palantir. From left to right, the average differentiation potential (DP) of cells at each developmental stage; UMAP displays the cells' DP (entropy), pseudotime, developmental stage and cell type.

3. A time-resolved analysis of gene regulatory networks (GRNs) or transcription factor (TF) modules could significantly enhance the study. For instance, in the pituitary epithelial lineage, Tub is known to play a role in pituitary function. The current analysis should either: demonstrate how mdCLA independently recapitulates Tub’s known functon(e.g., through stage-specific GRNs or

temporal expression dynamics), or shift emphasis to atlas-driven discoveries (e.g., identification of novel TFs enriched in specific pituitary epithelial subtypes).

Reply: We thank the reviewer for this valuable suggestion. In accordance with this feedback, we performed transcription factor (TF) co-expressed module analysis for epithelial cells (see Methods). By comparing the TF modules expression score across epithelial cell types, we found that the *Tub* module is one of the most enriched modules of pituitary gland (Response to Reviewer #2 Fig. 4a and Fig. 2e). Furthermore, an examination of the temporal dynamics of the top 30 modules for each epithelial cell type revealed that *Tub* activity is significantly upregulated during late-stage development (Response to Reviewer #2, Fig. 4b). These analyses illustrated that mdCLA enables atlas-driven identification of key transcriptional regulators governing epithelial differentiation.



Response to Reviewer #2 Figure 4. a. Rank plot showing the top TF modules of pituitary gland compared across 14 organs. Module scores were calculated by AUCell algorithm. Differentially expressed modules were identified by t-test, with thresholds set as adjusted p-value < 0.001 and $\log_2$ fold change > 1, colored in red. b. Heatmap showing the temporal expression trend of the top30 pituitary modules.

Regarding the role of *Tub* in the pituitary, we would like to clarify that its function in pituitary development has remained poorly characterized. While previous studies established that *Tub*-deficient mice exhibit late-onset obesity (PMID: 11801719), the underlying mechanism has been attributed primarily to the hypothalamus, where *Tub* is highly expressed. However, global knockout of *Tub* does not result in notable structural anomalies in the hypothalamus, suggesting that hypothalamic dysfunction alone may not fully account for the observed obesity phenotype (PMID: 36452038, PMID: 28852204). These observations prompted us to examine the potential role of *Tub* in pituitary gland. Through cross-organ comparative analysis of transcriptional regulatory programs, our study identified TUB as a TF specifically upregulated during late stages of pituitary development. Pituitary-specific conditional knockout experiments further confirmed a functional role for TUB in the pituitary gland, as its deletion resulted in increased body weight in mice (Fig. 5). Our study provides a new clue for understanding the obesity mechanism caused by *Tub* knockout and underscores mdCLA as a powerful resource for uncovering critical developmental events and

regulatory factors.

As this is primarily a resource paper, the authors should consider providing a more user-friendly interface for data access and visualization. This could include a searchable web portal, download-friendly metadata tables, and precomputed gene expression plots to facilitate broad reuse by the community.

Reply: We thank the reviewer for this helpful suggestion. We have upgraded the project website, making it more user-friendly. It now allows users to visualize gene expression plots and provides data download links. Users can access the website via the URL: http://mdcla.ccla.ac.cn/

Minor Issues

1. While the authors identify two NPC populations, their developmental origins and fates remain unclear. Is NPC2 derived from NPC1, or do they emerge from separate progenitors? Additionally, are these populations associated with distinct nephrogenic niches? The mention of immune-related GO terms in NPC2 is intriguing — could this suggest a microenvironmental influence or signaling niche that has not yet been characterized?

Reply: We thank the reviewer for raising these important questions. As described in our response to Reviewer #2 Figure 1, velocity analysis indicates that NPC6 (referred to as NPC2 in the previous version) is derived from NPC1.

To determine whether distinct NPC populations are associated with different nephrogenic niches, we performed spatial transcriptomic profiling of mouse kidneys at E14.5 and E18.0 using the 10x Genomics Xenium 5K platform (Response to Reviewer #2, Fig. 5a, b). This technology enables the detection of ~5,000 genes at single-cell resolution while preserving spatial information, allowing a direct assessment of cell states in their native tissue context.

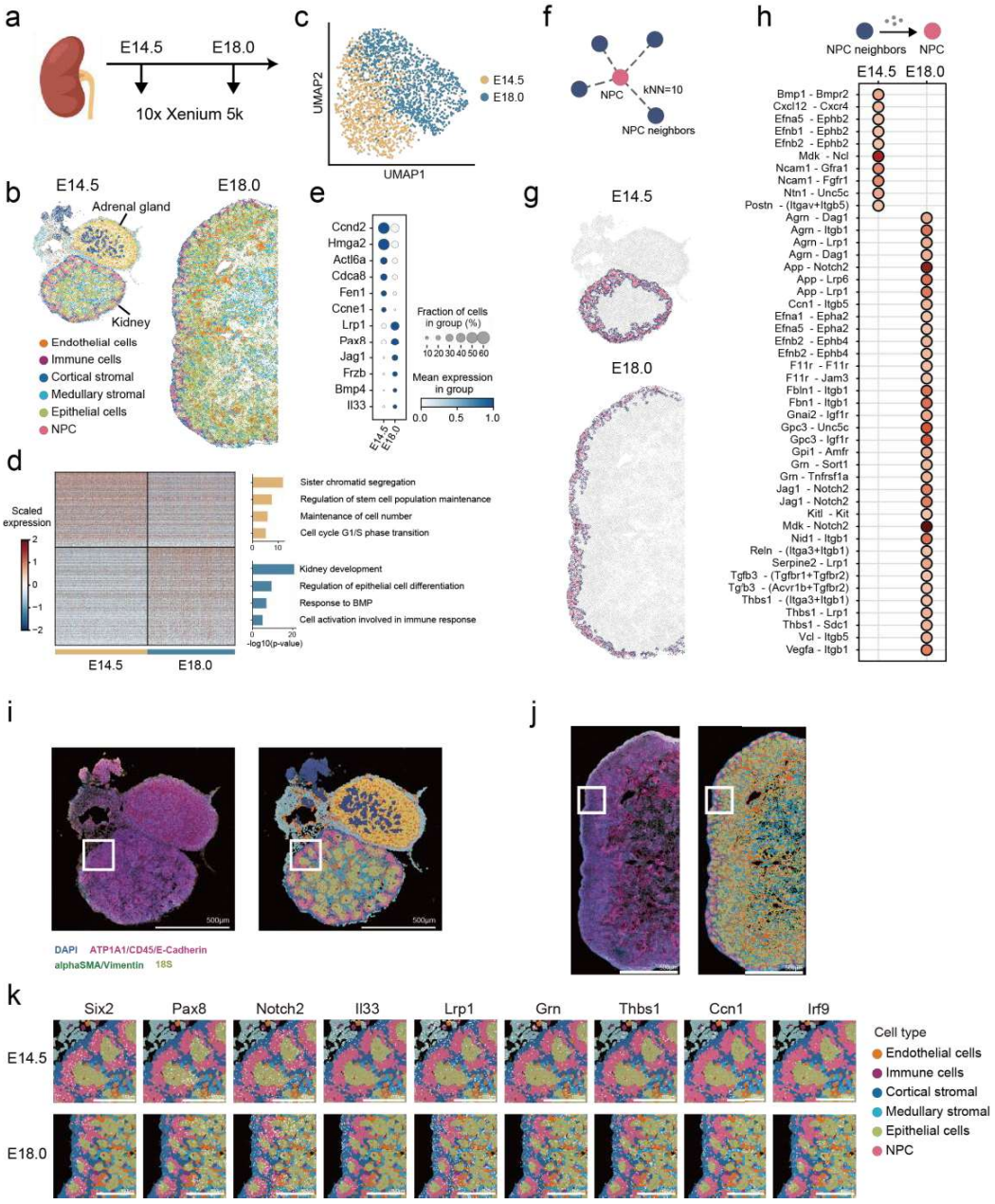
In NPC populations, genes related to mitotic nuclear division were downregulated at E18.0 (e.g., *Cdca8*). Genes associated with immune pathway signaling (e.g., *Il33*) were significantly upregulated at this stage (Response to Reviewer #2, Fig. 5c-e). Spatial data analysis results validate the findings from single-cell data.

We then identified the spatial neighbors of NPCs (k-nearest neighbors, kNN = 10) (Response to Reviewer #2 Figure 5f). We performed ligand-receptor interaction analysis between NPC neighbors and NPCs (Response to Reviewer #2 Figure 5g-h). Compared with E14.5, NPCs and their niches at E18.0 exhibited significant upregulation of pathways involved in epithelial differentiation and immune regulation. The Jag1-Notch2 and TGFβ pathways are known to critically regulate the epithelial differentiation of NPCs (PMID: 23806616, PMID: 29945868. We identified that *Il33*, *Ccn1* and *Thbs1* were highly expressed in E18.0 stromal cells, and their roles have not yet been characterized in previous studies. For example, cellular communication network factor 1 (CCN1) has been reported to regulate renal tissue regeneration in adult mice (PMID: 38598837), but its function in embryonic development remains unknown. We visualized the expression of differentially expressed genes (DEGs) on spatial tissue sections (Response to Reviewer #2 Figure

5i-k).

We next identified the spatial neighbors of NPCs using a k-nearest neighbors (kNN) approach (k = 10) (Response to Reviewer #2, Fig. 5f) and performed ligand–receptor interaction analysis between NPCs and their neighboring cells (Response to Reviewer #2, Fig. 5g, h). Compared with E14.5, NPCs and their neighbors at E18.0 exhibited significant upregulation of pathways involved in epithelial differentiation (Response to Reviewer #2, Fig. 5h). For example, the Notch pathway is known to critically regulate the epithelial differentiation of NPCs. Immune response related genes such as *Ccn1*, *Thbs1* and *Irf9* were also highly expressed in E18.0 niches (Response to Reviewer #2, Fig. 5i-k). Cellular communication network factor 1 (*Ccn1*) has been reported to regulate renal tissue regeneration in adult mice (PMID: 38598837), but its function in development in unknown. Interferon regulatory factor 9 (*Irf9*) is a core transcription factor in the type I interferon (IFN-I) signaling pathway, which forms a complex with STAT1/STAT2 to activate interferon-stimulated genes (ISGs) and orchestrate immune regulation (PMID: 39384770). The activation of *Irf9* suggests changes in the immune microenvironment of the kidney during the late gestation. These analyses indicate that NPCs heterogeneity may be associated with stage-specific niche signaling, linking progenitor cell states to dynamic microenvironmental regulation during kidney development.

Together, our study uncovers previously unrecognized components of the NPC niche during kidney development. To better present these findings, we have added a new Figure 3 that specifically focuses on NPC development, along with the corresponding descriptions in the revised manuscript.



Response to Reviewer #2 Figure 5. a. Spatial transcript profile using Xenium 5k technique. b. Cell type annotation of spatial data. c. UMAP showing the NPC populations from spatial data. d. Differentially expressed genes and GO analysis of NPCs between E14.5 and E18.0. e. Dot plot showing the differentially expressed genes between E14.5 and Eq18.0. f. schematic diagram illustrating the identification of NPC neighbor cells. g. Spatial layout showing the NPCs and NPC neighbor cells. Pink color indicates NPCs, blue color indicates NPC neighbors. h. The differential ligand-receptor interactions between NPCs and NPC neighbors at E14.5 and E18.0. Calculated by LIANA package. i-k. Spatial plot shows the gene expression in NPC niche.

2. Some figures require better explanation, especially Figure 3B (trajectory tree) and Figure 3C

(subtype timing). The current figure legends and main text descriptions are insufficient to interpret these panels independently.

Reply: We thank the reviewer for these valuable suggestions. We have revised the figure legends and the corresponding descriptions in the main text in the revised manuscript. We hope that these modifications improve the clarity of data presentation and effectively address the reviewer's concerns.

Point by point to the reviewers' comments

Reviewer #1:

I thank the authors for such a thorough revision of their manuscript "Developmental Chronology of Mouse Embryo" which I now believe is ready for publication. The detailed subsampling/saturation analysis is quite interesting, particularly highlighting the structural differences in transcript coverage and detection when using sci-RNA-seq3. The refined analyses related to transcription factor prediction, epithelial expansion and in situ validation of Nephron Progenitor Cell dynamics are also all very valuable, and I appreciate the more detailed comparison to Qui et al, 2024 in terms of coverage and performance. This is a very valuable and impressive resource.

We sincerely thank the reviewer for the highly positive and encouraging assessment of our revised manuscript. We are delighted that the reviewer considers our work to be a valuable and comprehensive resource for the field.

Reviewer #2:

We appreciate the authors' substantial efforts in the revision. The manuscript is materially improved and now includes additional experimental and bioinformatic validation that strengthens the central observations. We have no further concerns regarding the manuscript.

We sincerely thank the reviewer for the positive evaluation and for acknowledging the substantial improvements made in the revised manuscript. We are pleased that the reviewer has no further concerns.