

Induction and expansion of human PRRX1+ limb-bud-like mesenchymal cells from pluripotent stem cells

Corresponding author: Takeshi Takarada

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Fri 03 Apr 2020

Decision on Article nBME-20-0353

Dear Prof Takarada,

Thank you for submitting to *Nature Biomedical Engineering* your Article, "Induction and stable expansion of human pluripotent stem cell-derived PRRX1-positive limb bud mesenchymal cells capable of prospective assessment of chondrogenic potential". I regret that we are unable to consider it for external peer review.

As you may know, we screen manuscripts against our editorial criteria, and decline a substantial proportion of them without input from external referees. In such cases, even if reviewers were to certify the manuscript as technically correct, we feel that it would not be of outstanding interest to merit publication in *Nature Biomedical Engineering*. These editorial judgements are based on considerations of the degree of advance, broad implications, and breadth and depth of the work reported in the manuscript.

The topic of the Article is within the remit of the journal. However, in view of the work of others on hPSC-derived chondrocytes for cartilage regeneration, and in the absence of evidence that your strategy to derive intermediate chondrocytes can improve either the treatment of chondral defects as demonstrated in orthotopic animal models, or the efficiency of cell production in head-to-head comparisons with other state-of-the-art protocols, we feel that the degree of advance of the work does not reach the high threshold that we look for in manuscripts that we consider for external peer review. In addition, we feel that further insights into disease modelling of patients with type II collagenopathy is lacking. For us to consider the work we would need to see how the ExpLBMs could be leveraged to provide an advantage in the cell quality, numbers and cost efficiency for patient targeted interventions or further insights into disease modelling.

Nevertheless, the work may be appropriate for [Communications Biology](#) (a selective open-access journal from Nature Research), to which it could be transferred by using the link in the footnote below.

Please be assured that this editorial decision does not represent a criticism of the technical quality of the work. We hope that you will soon be able to see the manuscript published.

Best wishes,

Michelle

Dr Michelle Korda

Senior Editor, [Nature Biomedical Engineering](#)

* If you wish to transfer your manuscript to *Communications Biology*, please use our [manuscript transfer portal](#) to initiate the transfer to this journal (or to another journal of your choice published by Nature Research). This transfer link remains active until used. If you transfer your manuscript to Nature-branded journals or to the Communications journals, you will not have to re-supply manuscript metadata and files.

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Tue 05 Jan 2021

Decision on Article NBME-20-0353A-Z

Dear Prof Takarada,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Induction and stable expansion of human pluripotent stem cell-derived PRRX1-positive limb bud mesenchymal cells capable of prospective assessment of chondrogenic potential". The manuscript has been seen by 3 experts, whose reports you will find at the end of this message. You will see that the reviewers have good words for the work, and that they raise a number of technical criticisms that we hope you will be able to address. In particular, we would expect that a revised version of the manuscript provides:

*Comparison of the method to generate limb-specific progenitors to published methods, as suggested by Reviewer #1.

*Clarification of the meaning of the quality of the intermediate progenitors in the context of this study, as suggested by Reviewer #1.

*Further characterisation of cell phenotypes using RNA-sequencing and hypertrophic differentiation, as suggested by Reviewer #1 and #3.

*Quantification of survival rates of the graft, and tumourigenicity assays, as suggested by Reviewer #1.

*Clarification of cell surface markers for enrichment of limb-specific hyaline chondrogenesis and clarification of high chondrogenic differentiation using cell sorting, as suggested by Reviewer #1 and #3.

*Reproducibility of the data from more clones, as suggested by Reviewer #2.

*With regard to the presentation of statistics and in relation to Reviewer #2's comment, please use the following guidance:

Wherever statistics have been derived (e.g. error bars, box plots, statistical significance) the legend needs to provide and define the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), using the wording "n=X biologically independent samples/animals/cells/independent experiments/n=X cells examined over Y independent experiments" etc. as applicable.

We strongly discourage deriving statistics from technical replicates, unless there is a clear scientific justification for why providing this information is important. Conflating technical and biological variability, e.g., by pooling technically replicates samples across independent experiments is strongly discouraged. (For examples of expected description of statistics in figure legends, please see the following <https://www.nature.com/articles/s41467-019-11636-5> or <https://www.nature.com/articles/s41467-019-11510-4>).

Whilst it is not obligatory we suggest that the manuscript is copy edited or checked for English language errors prior to submission either by a native English speaker or using a professional editing service.

When you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) [reporting summary](#), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers with the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 25 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*. Because of the COVID-19 pandemic, should you be unable to carry out experimental work in the near future we advise that you reply to this message with a revision plan in the form of a preliminary point-by-point rebuttal to the comments from all reviewers that also includes a response to any points highlighted in this decision. We should then be able to provide you with additional feedback.

We hope that you will find the referee reports helpful when revising the work, which we look forward to receive. Please do not hesitate to contact me should you have any questions.

Best wishes,

Michelle

Dr Michelle Korda
Senior Editor, [Nature Biomedical Engineering](#)

Reviewer #1 (Report for the authors (Required)):

This study has the potential to become a milestone in the field of cartilage tissue engineering from human pluripotent stem cells, but a number of issues would need to be addressed to fully convince the readers. -Yamada and colleagues state that they have developed the first method to generate limb-specific hyaline cartilage from pluripotency using xeno-free conditions. To convince readers that their method is truly novel, they should compare their method to published ones and should highlight in which way their new method differs from others and guarantees generation of limb-specific progenitors (rather than other progenitor types).

-The authors start their abstract and study by claiming that “current protocols do not consider the quality of intermediate progenitors, which is required to achieve a clinically sufficient supply and enhance processability of tissue engineering”. It is not clear in the abstract and manuscript what “quality” means (is it phenotype?) and also how the authors know that this quality control step is required for both stated goals. As presented, it looks like the authors looked for a strong justification of their study, but omitted to provide legitimate support for it. Providing such information is required to justify their study.

-The goal of the study was to obtain limb-specific, stable articular cartilage. It is now well known that key properties of articular cartilage are not limited to having a “hyaline” aspect, expressing pancartilaginous markers (SOX5, SOX6, SOX9, COL2A1 and ACAN), and not expressing hypertrophic chondrocyte and fibroblast/osteoblast markers. Key articular cartilage properties also include expressing articular-specific markers (such as PRG4, CILP and CLU). This aspect was ignored by the authors. A more comprehensive phenotypic characterization of cell phenotypes (e.g., through RNA-seq profiling) is recommended.

-The authors wrote that they tested the graft survival and nontumorigenic properties of their newly derived cartilaginous particles. They, however, did not provide any quantification of the survival rates of graft (how many did they test and many did survive?) and did not do any tumorigenic assessment. These two critical

properties have therefore not been assessed rigorously.

-The authors tested the reproducibility of their new method using several iPSC lines. They should indicate how these lines were derived (what tissue/cell types were used and what the age and gender of the donors were) to indicate whether their protocol has been validated with a limited or a large spectrum of patient-derived iPSC lines.

-The authors looked for cell surface markers that would help distinguish PRRX1^{high} (limb-specific) and PRRX1^{low} limb-specific progenitor cells. They identified a CD90⁺CD140B⁺CD82⁻ signature for PRRX1^{high} cells. However, they missed to demonstrate that sorting cells for this signature does indeed allow enrichment of progenitor populations that have a higher potential than cells lacking this signature for limb-specific hyaline chondrogenesis. This interesting part of the study thus lacks proof of the proposed concept.

-The last part of the study is incomplete and unclear. "A" novel polymer was used to generate numerous cartilage particles with a uniform size. What is this polymer? Can the particles be used for tissue repair? This last part of the study is potentially interesting, but would probably be better presented in depth in a separate study.

Minor comments

-The authors claim in the Discussion that their new protocol allows for economically mass-producing cartilaginous tissues and that it is also less time-consuming than other protocols. These cost and time aspects are not substantiated in any way in the study. They should either be explained more explicitly or omitted.

-The Results section is overall overly concise, with many concepts and reagents lacking proper introduction, and without full descriptions of goals, methods and results. It is therefore difficult to follow, even for experts in the field.

-The text is also often difficult to understand because of English language issues (including improper use of the "the" article).

Reviewer #2 (Report for the authors (Required)):

In this manuscript, the authors reported a stepwise protocol to differentiate human pluripotent stem cells (hPSCs) into chondrocytes. First, the authors established a PRRX1-tdTomato-reporter hPSCs, which was applied to optimize the protocol to derive xeno-free ontogenetically-defined, expandable PRRX1-positive limb bud mesenchymal (ExpLBM) cells, which stably proliferated and exhibited the capability to differentiate into chondrocytes both in vitro and in vivo. The stepwise protocol was further applied to patient derived iPSCs and show that both ACGII-1 iPSCs and HCG-1 iPSCs show impaired abilities to differentiate into LBM cells. Then, a high content chemical screen was applied to identify the drug candidate that can potentially rescue the impaired chondrogenic capacities of ACGII-iPSCs. Finally, a self-aggregation inducible specific-charged polymer was applied to generate relatively homogenous LBM cells for replacement therapy and drug screen. Overall, this manuscript reported a new reporter for chondrocyte differentiation and a detailed differentiation protocol. The protocol was described in details, which potentially have a significant impact to the field. The manuscripts were well-organized with a large amount of data. Here are some points that need to be addressed before considered for publication.

1. Considering the clone to clone variations, two iPSC clones from each patient iPSC lines should be applied to validate the phenotype.

2. Some data were presented in a confusing way. For example, in Fig 1h (and all figure panels showing quantification), three data points are presented. However, the figure legends showed n = 3, two biologically independent experiments. Does this figure panel show 3 technical replicates of one biologically independent experiments? For all experiments, minimal three biologically independent experiments are required.

3. The main text is lack of rationale and experimental details.

Reviewer #3 (Report for the authors (Required)):

This study addresses highly relevant questions regarding technology development to generate intermediate PRRX1-positive limb bud mesenchymal cells that can be expanded and differentiated to chondrocytes for applications in cartilage tissue repair, mechanistic studies and drug screening.

The notion that PRRX1-positive limb bud mesenchymal cells are a potentially promising cell source is supported by prior studies and validated by tracing these cells following culture in the presence of various agents which affect signal transduction, or with recombinant proteins.

Overall, most results are solid.

Two key issues need to be addressed:

1. More thorough analysis of hypertrophic differentiation.

2. Demonstrating that cell sorting with the candidate markers does indeed confirm the hypothesis that these markers will deliver a cell population of ExplBM cells with high chondrogenic differentiation potential.

Other specific suggestions:

General comment: Time points when analyses were performed should be mentioned in the legends to all Figures and supplemental figures.

Lines 129-136: at least some level of detail should be provided to explain the RNAseq results and mention key differences between the time points.

Line 162: Mention whether PCR showed ColX or other hypertrophy markers. The 6 day time point is too early to assess hypertrophy markers, including IHC for COLX

Assessment for hypertrophy markers should also be included in Sup. Fig.6 and Sup. Fig. 7

Lines 207-210: While the RNAseq revealed promising cell surface receptors, this has not been validated by cell sorting. This should be done.

Wed 26 May 2021

Decision on Article NBME-20-0353A-Z

Dear Prof Takarada,

Thank you for your revised manuscript, "Induction and stable expansion of human pluripotent stem cell-derived PRRX1-positive limb bud mesenchymal cells", which has been seen by the original Reviewers #1 and #3. In their reports, which you will find at the end of this message, you will see that the reviewers acknowledge the improvements to the work and that Reviewer #1 raises a few additional criticisms that we hope you will be able to address. In particular, we would expect that the next version of the manuscript provides evidence of the phenotypes of the chondrocytes generated, and clarifies the differences with previous protocols. Also, please do take this opportunity to revise the entire text to improve its clarity, technical accuracy and flow, as the reviewer recommends.

As before, when you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from Reviewer #1, the (revised, if needed) [reporting summary](#), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

We look forward to receive a further revised version of the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

Pep Pàmies
Chief Editor, [Nature Biomedical Engineering](#)

Reviewer #1 (Report for the authors (Required)):

The authors have significantly improved their manuscript and data by appropriately addressing most reviewers' comments. I would still like them to address the following points.

Major comments

- It is unclear what the authors meant to say when they wrote that current protocols "do not consider the differentiation capacity of intermediate progenitors" and that these protocols "lack the ability to evaluate and control the quality of transitional stages". This is an important point in assessing the novelty and reliability of their protocol compared to others. Did they mean that intermediate cells were not tested or did they mean that these cells were not testable? The text should be edited and more explanation should be provided to make this point crystal-clear.
- The authors now indicate the composition of the novel zwitterionic copolymers used to make cartilaginous tissues. The safety and resorbability of these copolymers in vivo should be at least commented on, if not demonstrated.
- In Discussion, the authors still mention that their protocol "achieves cost-effective mass productivity and high processability". A protocol that requires generation and characterization of hiPSCs does not seem to us more cost-effective than protocols using hMSCs. Authors should explain this further or omit this aspect from their manuscript.
- It remains unclear as whether the new protocol generates growth plate or articular chondrocytes or a mixture of both cell types. This is an important point to address experimentally and in the text, as growth plate chondrocytes are not desirable for articular cartilage repair.

- In Discussion, the authors now refer to a new Supplementary Table 4 to compare their protocol to previous ones. They should explicitly describe differences between protocols in their text. In addition, they indicated that other protocols lead to “insufficient numbers of cells and low reproducibility”. What did they base these comments on?

Minor comments

- The authors should be more cautious in naming their induced cells “limb bud mesenchymal cells” in the title and elsewhere in the text. The work “limb bud-like” would be more appropriate.

- The last paragraph of the introduction should be considerably shortened to briefly describe the main messages of this paper. The present tense could be used for this paragraph.

- First section of Results. At this stage in the paper, the authors have not demonstrated yet that their induced cells are really LBM-like. Therefore, LBM should be omitted from the header of this section and the text edited as appropriate.

- The second section of Results should be entitled “Expansion and characterization of LBM-like cells under xeno-free, defined conditions”, since LBM features are tested here (rather than in the first section).

- The text still needs further editing. We recommend the authors to seek advice from an expert in the iPSC/chondrocyte field who is a native-english speaker, because many sentences are scientifically inaccurate or difficult to understand, likely because of English language issues. Also, authors should omit the use of pleonasms, which make the text confusing to read (for instance, the words robust should be deleted in the phrase “robust mass production” as it is redundant with “mass”).

- Figure 1a. Authors should provide all information necessary to understand how tdTomato was knocked into the PRRX1 locus. For the PRRX1 gene schematic, authors should indicate that only the last two exons are shown. For the targeting vector, they should indicate whether homology arms were included 5' and 3' of the Tomato/NeoR cassette and whether the primers used for PCR screening were within or outside the homology arms. It appears from the schematic that the primers may have been located within the homology arms, which would not be appropriate.

Reviewer #3 (Report for the authors (Required)):

Authors provided satisfactory revisions to address reviewer's comments.

Wed 23 Jun 2021

Decision on Article NBME-20-0353A-Z

Dear Prof Takarada,

Thank you for your revised manuscript, "Induction and stable expansion of human pluripotent stem cell-derived PRRX1-positive limb bud-like mesenchymal cells". Having consulted with Reviewer #1 (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please [upload](#) the files specified in the instructions file.

For primary research originally submitted after December 1, 2019, we encourage authors to take up [transparent peer review](#). If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons. If any reviewers have signed their comments to authors, or if any reviewers explicitly agree to release their name, we will include the names in the peer-review supplementary file. [More information on transparent peer review is available.](#)

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

Pep Pàmies
Chief Editor, [Nature Biomedical Engineering](#)

P.S. Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol-sharing platform of their choice. Nature Research's [Protocol Exchange](#) is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can be found at www.nature.com/protocolexchange/about.

Reviewer #1 (Report for the authors (Required)):

The authors have now reasonably well responded to my previous critiques.

English language remains suboptimal in many sentences. In particular. I still don't understand what the authors are trying to say in the first sentence of the abstract. Do they actually mean to say that "current protocols for the differentiation of human pluripotent stem cells (hPSCs) into chondrocytes do not include methods to assess the chondrogenic potential of intermediate progenitors"?

Similarly, the new sentence added in the introduction: "however, methods to expand transitional lineage (i.e. progenitor) cells capable of prospective assessment of chondrogenic potential have not yet been developed" does not seem to convey the actual message of the authors. I believe that the authors meant to say:

"however, none of these methods include quality tests to determine whether intermediate progenitor cells exhibit bona fide phenotypic features of chondrogenic progenitors in vivo".

Page 2, line 27, the text should be "... with primers that recognize sequences flanking the homology arms (HA) identified a clone harboring a homozygous knock-in of the reporter cassette." Similarly, in the legend of figure 1, the new words should be changed to "... primers that recognize sequences outside the homology arms...".

Rebuttal 1

September 8th, 2020

Dr. Michelle Korda
Associate Editor
Nature Biomedical Engineering

Dear Dr. Michelle Korda,

We thank for your meaningful comments regarding our manuscript (nBME-20-0353) that has been submitted for consideration for publication as a Research Article in *Nature Biomedical Engineering*.

In order to clarify the differences between our chondrogenic protocol and that of other groups, we prepared a comparative table that shows the critical points of each published method (Table 1). Compared to other protocols, our method is superior in the following respects: 1. Expandable or cryopreservable progenitor resource for cartilage regeneration, 2. No requirement of cell sorting for obtaining the expandable/cryopreservable progenitors, and 3. Identification of cell-surface markers for prospective assessment of their chondrogenic potential. These points have been listed in comparison with other protocols as Supplementary Table 6 in the revised manuscript.

Table 1 | Comparison of chondrogenic protocols

	Our method	Method A Stem Cell Reports (2015) 4:712-726.	Method B Nat Biotechnol (2015) 33:638-645.	Method C Stem Cell Reports (2015) 4:404-418.	Method D Stem Cell Reports (2019) 13:530-544.
Cell source	hiPSC or hESC	hiPSC or hESC	hiPSC or hESC	hiPSC or hESC	hiPSC or hESC
Intermediate state	Lateral plate mesoderm / Limb bud mesenchyme	Neural crest / Ectomesenchyme	Paraxial mesoderm	Mesendodermal	Mesendodermal
Expansion of progenitor cells	Yes	Yes	No	No	No
Cell sorting during production of progenitor cells	Unnecessary	CD271 ⁺ / CD73 ⁻ fraction should be isolated after inducing neural crest-like cells.			
Cryopreservation of progenitor cells in liquid nitrogen	Yes	Not described			
Purity of progenitor cells	Homogeneous (PRRX1 ⁺ / SOX9 ⁺ = 100%)	Heterogeneous (SOX9 ⁺ = 80 %)			
Surface antigens to assess the quality of progenitor cells before chondrogenic induction	CD90 high, CD140B high, CD82 ⁻	CD271 ⁺ , CD140A ⁺ , CD73 ⁺ , CD13 ⁻ , CDH2 ⁻			
Xeno-free chondrogenic induction	Yes	No	Yes	No	Yes

As mentioned in your comments, we also confirmed the engraftment of our cartilaginous particles to a 1-mm diameter hole in articular cartilage of SCID rats, corroborating their application for regenerative medicine (Figure 1). These data have been added as Figure 3 in the revised manuscript.

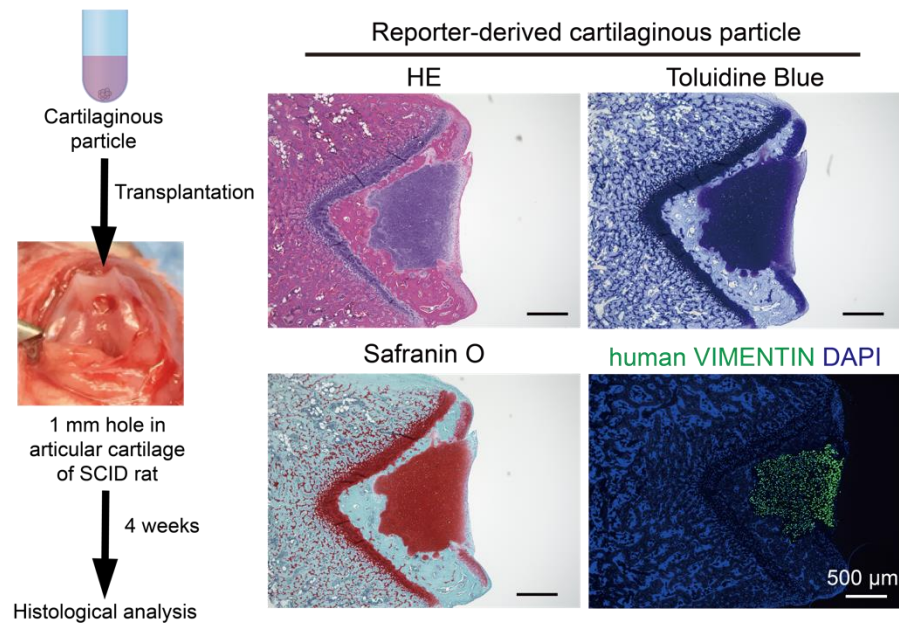


Figure 1 | Assessment of the regenerative capacity of ExpLBM-derived cartilaginous particle
Engraftment of cartilaginous particles in damaged articular cartilage of SCID rats. We formed 1-mm drill holes in the articular cartilage of SCID rats, and then PRRX1³tdTomato-reporter-derived cartilaginous particles were embedded. The knee joints were fixed at 4 wk after transplantation, and tissue sections were stained with hematoxylin and eosin, toluidine blue, or human VIMENTIN. Representative images are shown.

Moreover, we established a novel chemical screening system based on our two-dimensional

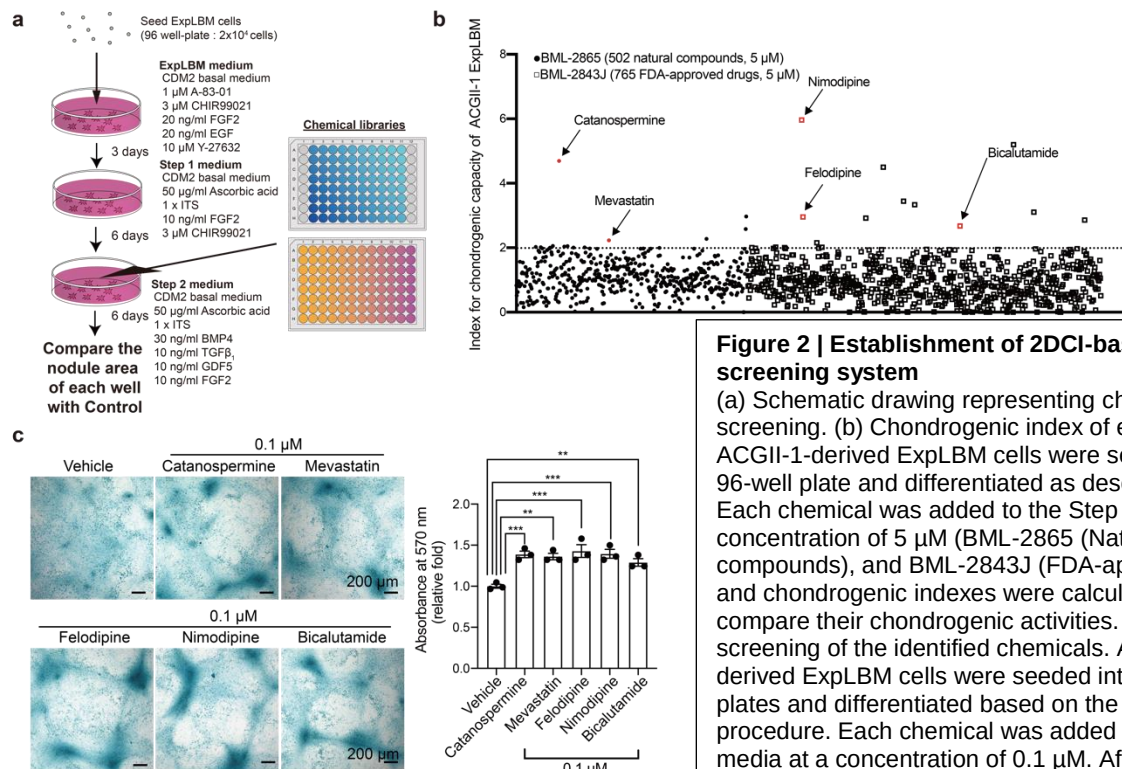
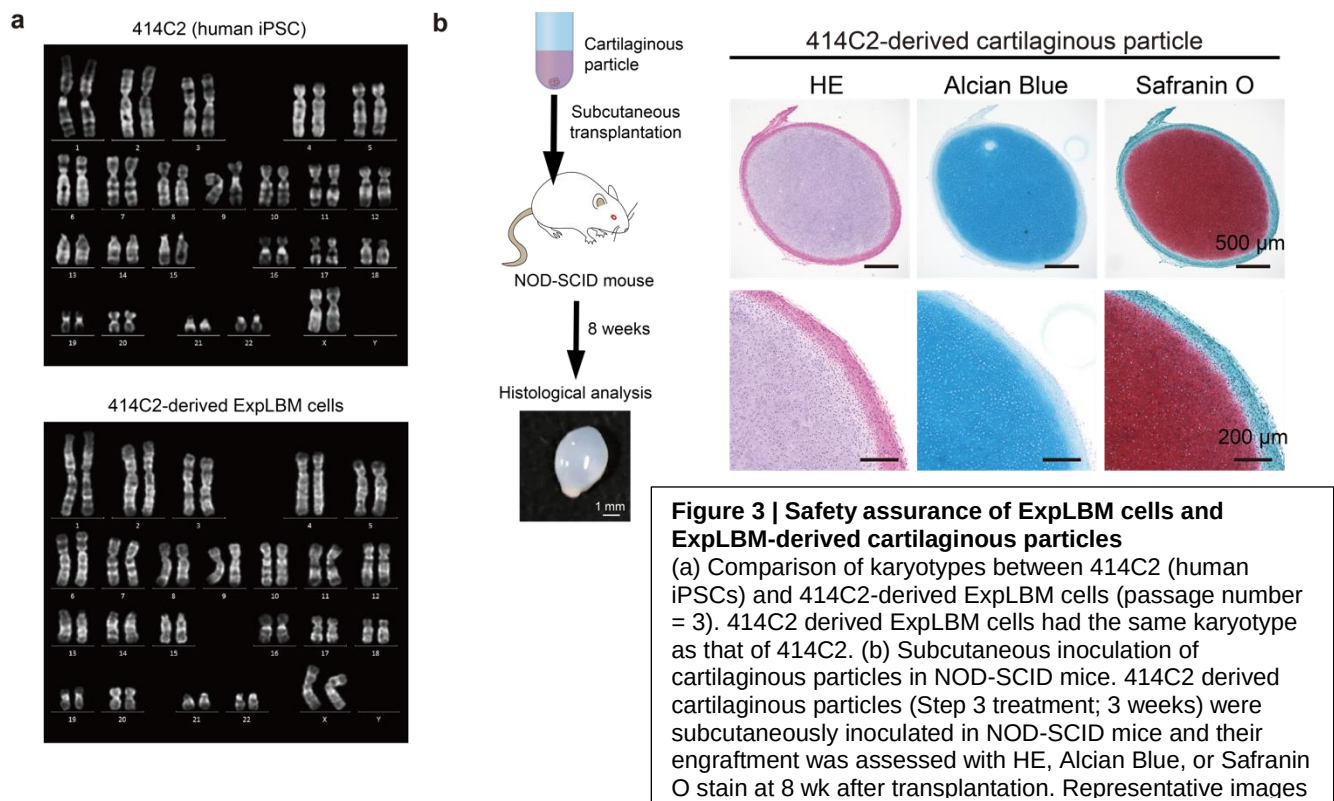


Figure 2 | Establishment of 2DCI-based chemical screening system
(a) Schematic drawing representing chemical screening. (b) Chondrogenic index of each chemical. ACGII-1-derived ExpLBM cells were seeded into a 96-well plate and differentiated as described above. Each chemical was added to the Step 2 media at a concentration of 5 μM (BML-2865 (Natural compounds), and BML-2843J (FDA-approved drugs)) and chondrogenic indexes were calculated to compare their chondrogenic activities. (c) Second screening of the identified chemicals. ACGII-1-derived ExpLBM cells were seeded into 96-well plates and differentiated based on the indicated procedure. Each chemical was added to the Step 2 media at a concentration of 0.1 μM. After culturing with Step 2 media for 6 days, the cells were fixed, and Alcian Blue staining was performed (n = 3, two independent experiments). Representative images are shown. *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

chondrogenic induction (2DCI) protocol and found that several chemicals among our chemical libraries promoted the chondrogenic differentiation of ACGII-1 (COL1pathy patient-derived iPSC)-derived ExpLBM cells (Figure 2). These data have been added as Figure 5 in the revised manuscript.

Furthermore, we confirmed that ExpLBM cells maintained normal karyotype after several passages (Figure 3a) and ExpLBM-derived cartilaginous particles did not form any tumor-like tissues at 8 wk following their subcutaneous transplantation (Figure 3b and 3c). These data were have been added as Figure 3 or Supplementary Figure 3 in the revised manuscript.



Our data demonstrate that ExpLBM cells possess regenerative potential, are safe regenerative resources of the hyaline cartilage, and are specialized for 2DCI-based chemical screening system or disease modeling. We believe that our paper is now suitable for publication in *Nature Biomedical Engineering*. We look forward to your reply.

Sincerely yours,

Takeshi Takarada, Ph.D.

Enc.

Rebuttal 2

Replies to Reviewer #1:

1. Yamada and colleagues state that they have developed the first method to generate limb-specific hyaline cartilage from pluripotency using xeno-free conditions. To convince readers that their method is truly novel, they should compare their method to published ones and should highlight in which way their new method differs from others and guarantees generation of limb-specific progenitors (rather than other progenitor types).

Response:

We thank the reviewer for this suggestion to corroborate the novelty of our study. In [Supplementary Table 4](#), a comparison of the methods used to induce chondrogenic progenitor cells from human pluripotent stem cells is provided, indicating that the published chondrogenic methods were not based on induction from human limb-specific progenitors. In addition, NGS analysis found that our ExpLBM cells showed upregulation of LBM markers and downregulation of LPM or pluripotent markers ([Figure 2h](#)). These results demonstrated that our method generates hyaline cartilage exclusively via human limb-specific progenitors. To clearly demonstrate the novelty of our findings, the Discussion section (page 7, lines 30-33; page 7, lines 43-46) was revised.

2. The authors start their abstract and study by claiming that “current protocols do not consider the quality of intermediate progenitors, which is required to achieve a clinically sufficient supply and enhance processability of tissue engineering”. It is not clear in the abstract and manuscript what “quality” means (is it phenotype?) and also how the authors know that this quality control step is required for both stated goals. As presented, it looks like the authors looked for a strong justification of their study, but omitted to provide legitimate support for it. Providing such information is required to justify their study.

Response:

We thank the reviewer for pointing this out because it is indeed difficult to understand the meaning of quality. In this study, as suggested by Reviewer #1, we defined quality as the differentiation capacity of progenitor cells. The manuscript was revised accordingly (page 1, line 7).

3. The goal of the study was to obtain limb-specific, stable articular cartilage. It is now well known that key properties of articular cartilage are not limited to having a “hyaline” aspect, expressing pancartilaginous markers (SOX5, SOX6, SOX9, COL2A1 and ACAN), and not expressing hypertrophic chondrocyte and fibroblast/osteoblast markers. Key articular cartilage properties also include expressing articular-specific markers (such as PRG4, CILP and CLU). This aspect was ignored by the authors. A more comprehensive phenotypic characterization of cell phenotypes (e.g., through RNA-seq profiling) is recommended.

Response:

We thank the reviewer for indicating this extremely important point. In response to this comment, we performed additional RNA-seq analysis of the differentiated samples (2DCI and 3DCI) from ExpLBM, followed by marker analysis. These new data are presented as [Supplementary Figure 5b](#) and [Supplementary Table 2](#) in the revised manuscript. Accordingly, several sentences have been added in the Results section (page 4, lines 23-30) and figure legends (page 35, lines 2-9).

4. The authors wrote that they tested the graft survival and nontumorigenic properties of their newly derived cartilaginous particles. They, however, did not provide any

quantification of the survival rates of graft (how many did they test and many did survive?) and did not do any tumorigenic assessment. These two critical properties have therefore not been assessed rigorously.

Response:

We appreciate this important comment. We transplanted cartilage particles into a subcutaneous site of SCID mice or into injured articular cartilage of SCID rats in this study. Accordingly, we added a summary table regarding the graft survival rate and teratoma incidence among the transplanted animals ([Supplementary Figure 8a](#)). All transplants were successfully engrafted, and no detectable teratoma formation was observed. In addition, we performed a colony forming assay to check the tumorigenicity of ExpLBM-derived cartilage tissues, which did not yield any detectable colonies ([Supplementary Figure 8b](#)). Accordingly, several sentences have been revised (page 4, line 41-47).

5. The authors tested the reproducibility of their new method using several iPSC lines. They should indicate how these lines were derived (what tissue/cell types were used and what the age and gender of the donors were) to indicate whether their protocol has been validated with a limited or a large spectrum of patient-derived iPSC lines.

Response:

We thank the reviewer for this comment. We added [Supplemental Table 5, which provides](#) detailed information about the human iPSC lines used in this study. Accordingly, several sentences have been added in the Methods (page 6, line 16).

6. The authors looked for cell surface markers that would help distinguish PRRX1^{high} (limb-specific) and PRRX1^{low} limb-specific progenitor cells. They identified a CD90⁺CD140B⁺CD82⁻ signature for PRRX1^{high} cells. However, they missed to demonstrate that sorting cells for this signature does indeed allow enrichment of progenitor populations that have a higher potential than cells lacking this signature for limb-specific hyaline chondrogenesis. This interesting part of the study thus lacks proof of the proposed concept.

Response:

We thank the reviewer for raising this extremely important point. To address this issue, we purified CD90^{high}CD140B^{high}CD82^{low} or CD90^{low}CD140B^{low}CD82^{high} populations from 414C2-derived ExpLBM cells using a cell sorter, followed by analyzing the chondrocyte differentiation capacity using Alcian blue staining and qPCR. These new data are presented in [Figure 4h](#) and [4i](#) in the revised manuscript. Accordingly, several sentences have been added in the Results section (page 5, lines 38-42) and figure legends (page 24, lines 16-19).

7. The last part of the study is incomplete and unclear. “A” novel polymer was used to generate numerous cartilage particles with a uniform size. What is this polymer? Can the particles be used for tissue repair? This last part of the study is potentially interesting, but would probably be better presented in depth in a separate study.

Response:

We appreciate this comment. We used zwitterionic copolymers that comprised *N,N*-dimethylaminoethyl methacrylate (DMAEMA) and methacrylic acid (MA) (PDMAEMA-co-PMA; Mn: 9.7×10^4 g/mol; PDMAEMA/PMA ratio: 10). In addition, we confirmed the successful engraftment of the cartilaginous particles generated using this zwitterionic copolymers in damaged articular cartilage of SCID rats. These new data are presented as [Figure 6f](#) in the revised manuscript. Accordingly, several sentences have been added in the Results section (page 7, lines 4-8), Methods section (page 12, lines 48-50) and figure legends (page 28, lines 14-17).

Minor comments

8. The authors claim in the Discussion that their new protocol allows for economically mass-producing cartilaginous tissues and that it is also less time-consuming than other protocols. These cost and time aspects are not substantiated in any way in the study. They should either be explained more explicitly or omitted.

Response:

We completely agree with this comment. The description of the cost and time aspects was omitted, and the manuscript has been revised accordingly (page 8, lines 21-23).

9. The Results section is overall overly concise, with many concepts and reagents lacking proper introduction, and without full descriptions of goals, methods and results. It is therefore difficult to follow, even for experts in the field.

Response:

We thank the reviewer for this important comment. As suggested, we added this information in Results (page 3, lines 33-47; page 5, lines 6–10; page 5 lines 49-50 - page 6 line 1) sections.

10. The text is also often difficult to understand because of English language issues (including improper use of the “the” article).

Response:

We thank the reviewer for this comment. Our manuscript has been revised by an English editing service and all grammatical errors have been corrected.

Replies to Reviewer #2:

1. Considering the clone to clone variations, two iPSC clones from each patient iPSC lines should be applied to validate the phenotype.

Response:

We thank the reviewer for this extremely important comment. In response to this comment, we obtained other ACGII-1 or HCG-1 iPSC clones derived from the same patients and tested their chondrocyte differentiation capacity using our 2DCI protocol. These data is added in [Supplementary Figure 10a](#) and the manuscript was revised accordingly (page 6, line 16).

2. Some data were presented in a confusing way. For example, in Fig 1h (and all figure panels showing quantification), three data points are presented. However, the figure legends showed n = 3, two biologically independent experiments. Does this figure panel show 3 technical replicates of one biologically independent experiments? For all experiments, minimal three biologically independent experiments are required.

Response:

We greatly appreciate Reviewer #2's comment regarding our careless mistakes. As we performed three independent experiments to prepare samples, the descriptions in each figure legend have been corrected.

3. The main text is lack of rationale and experimental details.

Response:

We thank the reviewer for this important comment. As suggested, we revised the Results section (page 3, lines 33-47; page 5, lines 6-10; page 5 lines 49-50 - page 6 line 1).

Replies to Reviewer #3:

1. More thorough analysis of hypertrophic differentiation.

Response:

We thank the reviewer for raising this extremely important point. In response to this comment, we performed additional RNA-seq analysis of differentiated samples (2DCI and 3DCI) from ExpLBM, followed by the analysis of hypertrophic markers. These new data are presented as [Supplementary Figure 5b](#) and [Supplementary Table 2](#) in the revised manuscript. In addition, we also performed qRT-PCR analysis of several hypertrophic markers, as presented in [Supplementary Figure 5a](#). Accordingly, several sentences have been added in the Results section (page 4, lines 23-30) and figure legends (page 35, lines 2-9).

2. Demonstrating that cell sorting with the candidate markers does indeed confirm the hypothesis that these markers will deliver a cell population of ExpLBM cells with high chondrogenic differentiation potential.

Response:

We thank the reviewer for raising this extremely important point. To address this issue, we purified CD90^{high}CD140B^{high}CD82^{low} or CD90^{low}CD140B^{low}CD82^{high} populations from 414C2-derived ExpLBM cells using a cell sorter, followed by the analysis of the chondrocyte differentiation capacity using Alcian blue staining and qPCR. The new data are presented in [Figure 4h](#) and [4i](#) in the revised manuscript. Accordingly, several sentences have been added in the Results section (page 5, lines 38-42) and figure legends (page 24, lines 16-19).

Other specific suggestions:

3. General comment: Time points when analyses were performed should be mentioned in the legends to all Figures and supplemental figures.

Response:

We thank the reviewer for this comment pointing out the lack of important information. Accordingly, we added this information in each figure legend (page 18, line 14; page 18, line 22; page 18, line 26; page 18, line 36; page 22, line 13; page 26 lines 18-19; page 30, lines 7-8; page 32, line 5).

4. Lines 129-136: at least some level of detail should be provided to explain the RNAseq results and mention key differences between the time points.

Response:

We thank the reviewer for this comment pointing out the lack of important information. As suggested, several sentences have been modified in the Results section (page 3, lines 33-47).

5. Line 162: Mention whether PCR showed ColX or other hypertrophy markers. The 6 day time point is too early to assess hypertrophy markers, including IHC for COLX assessment for hypertrophy markers should also be included in Sup.Fig.6 and Sup.Fig.7.

Response:

According to this comment, we performed qRT-PCR analysis of several hypertrophic markers as presented in [Supplementary Figure 5a](#). In addition, we also performed IHC analysis for COLX expression in [Supplementary Figure 7](#) and [Supplementary Figure 9](#) in the revised manuscript. Accordingly, several sentences have been added in the Results section (page 4, lines 37-39; page 4, lines 47-50) and Figure legends (page 38, line 9; page 40, line 6).

6. Lines 207-210: While the RNAseq revealed promising cell surface receptors, this has not been validated by cell sorting. This should be done.

Response:

We thank the reviewer for raising this extremely important point. To address this issue, we purified CD90^{high}CD140B^{high}CD82^{low} or CD90^{low}CD140B^{low}CD82^{high} populations from 414C2-derived ExpLBM cells using a cell sorter, followed by the analysis of the chondrocyte differentiation capacity using Alcian blue staining and qPCR. The new data were presented in [Figure 4h](#) and [4i](#) in the revised manuscript. Accordingly, several sentences have been added in the Results section (page 5, lines 38-42) and figure legends (page 24, lines 16-19).

Rebuttal 3

Replies to Reviewer #1:

1. It is unclear what the authors meant to say when they wrote that current protocols “do not consider the differentiation capacity of intermediate progenitors” and that these protocols “lack the ability to evaluate and control the quality of transitional stages”. This is an important point in assessing the novelty and reliability of their protocol compared to others. Did they mean that intermediate cells were not tested or did they mean that these cells were not testable? The text should be edited and more explanation should be provided to make this point crystal-clear.

Response: We thank the reviewer for this comment. To clarify these points, we have revised the sentences indicated by the reviewer as follows: “Current protocols for the differentiation of human pluripotent stem cells (hPSCs) into chondrocytes do not include methods to expand intermediate progenitors capable of prospective assessment of chondrogenic potential.” (page 1, line 5-7) and “methods to expand transitional lineage (i.e. progenitor) cells capable of prospective assessment of chondrogenic potential have not yet been developed.” (page 1, line 35-36)

2. The authors now indicate the composition of the novel zwitterionic copolymers used to make cartilaginous tissues. The safety and resorbability of these copolymers in vivo should be at least commented on, if not demonstrated.

Response: We thank the reviewer for this comment and have added a description on the safety of zwitterionic copolymers to the Materials and Methods section (page 13, line 8-9).

3. In Discussion, the authors still mention that their protocol “achieves cost-effective mass productivity and high processability”. A protocol that requires generation and characterization of hiPSCs does not seem to us more cost-effective than protocols using hMSCs. Authors should explain this further or omit this aspect from their manuscript.

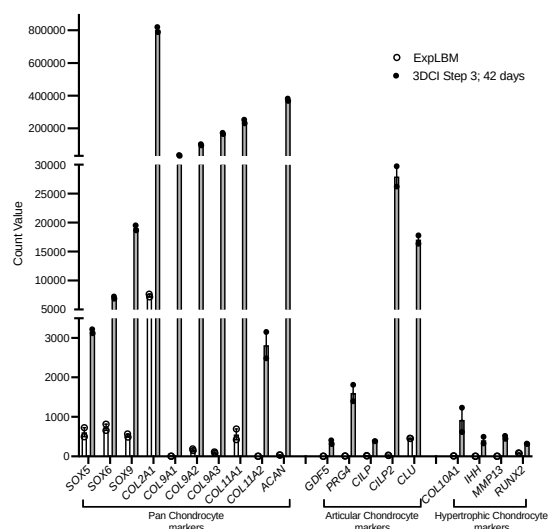
Response: We have removed this sentence from the manuscript.

4. It remains unclear as whether the new protocol generates growth plate or articular chondrocytes or a mixture of both cell types. This is an important point to address experimentally and in the text, as growth plate chondrocytes are not desirable for articular cartilage repair.

Response: We thank the reviewer for this comment because we believe that the cellular identity corroborates the novelty of our study. The count values obtained from the RNA-seq analysis of 3DCI-treated ExpLBM (step 3: 42 days) show that ExpLBM-derived cartilaginous tissues are composed of a mixture of articular and hypertrophic chondrocytes, suggesting that ExpLBM cells can generate both articular cartilage and growth plate cartilage tissues.

[REDACTED]

We have added this data (Supplementary Figure 5c) and description in the Discussion section (page 7, line 22-26) and have revised the manuscript accordingly.



5. In Discussion, the authors now refer to a new Supplementary Table 4 to compare their protocol to previous ones. They should explicitly describe differences between protocols in their text. In addition, they indicated that other protocols lead to “insufficient numbers of cells and low reproducibility”. What did they base these comments on?

Response: We thank the reviewer for the comment and have corrected the description difficult to understand accordingly (page 8, line 4-8).

6. The authors should be more cautious in naming their induced cells “limb bud mesenchymal cells” in the title and elsewhere in the text. The work “limb bud-like” would be more appropriate.

Response: We have changed “limb bud mesenchymal cells” to “limb bud-like mesenchymal (or LBM-like) cells” in the title and manuscript text.

7. The last paragraph of the introduction should be considerably shortened to briefly describe the main messages of this paper. The present tense could be used for this paragraph.

Response: We have corrected the last paragraph of the Introduction accordingly (page 2, line 6-14).

8. First section of Results. At this stage in the paper, the authors have not demonstrated yet that their induced cells are really LBM-like. Therefore, LBM should be omitted from the header of this section and the text edited as appropriate.

Response: As instructed, we have omitted the word “LBM” in the first section of the Results.

9. The second section of Results should be entitled “Expansion and characterization of LBM-like cells under xeno-free, defined conditions”, since LBM features are tested here (rather than in the first section).

Response: We thank the reviewer for the comment and have corrected the subtitle accordingly.

10. The text still needs further editing. We recommend the authors to seek advice from an expert in the iPSC/chondrocyte field who is a native-english speaker, because many sentences are scientifically inaccurate or difficult to understand, likely because of English language issues. Also, authors should omit the use of pleonasms, which make the text confusing to read (for instance, the words robust should be deleted in the phrase “robust mass production” as it is redundant with “mass”).

Response: We agree that detailed and accurate descriptions should be provided to allow readers to reproduce and understand our results. We have corrected several sentences mentioned by Reviewer #1 and have employed an English language editing service (with experts in the iPSC/chondrocyte fields).

11. Figure 1a. Authors should provide all information necessary to understand how tdTomato was knocked into the PRRX1 locus. For the PRRX1 gene schematic, authors should indicate that only the last two exons are shown. For the targeting vector, they should indicate whether homology arms were included 5’ and 3’ of the Tomato/NeoR cassette and whether the primers used for PCR screening were within or outside the homology arms. It appears from the schematic that the primers may have been located within the homology arms, which would not be appropriate.

Response: We agree that detailed information about our targeting strategy has not been

described in Figure 1a. The illustration and legend of Figure 1a has been corrected (page 18, line 8), and the manuscript has been revised accordingly to address the reviewer's concerns.