THE EMBO JOURNAL

Bronchioalveolar stem cells are a main source for regeneration of distal lung epithelia in vivo

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Review timeline:

Submission date:	25th Mar 2019
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Authors' Correspondence:	1st Apr 2019
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Editor: Daniel Klimmeck

Transaction Report:

(Please note that the manuscript was previously reviewed at another journal and the reports were taken into account in the decision making process at The EMBO Journal. Since the original reviews are not subject to EMBO's transparent review process policy, the reports and author response cannot be published here.
Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 1st Apr 2019

Thank you again for the submission of your amended manuscript (EMBOJ-2019-102099) to The EMBO Journal. We have carefully assessed your manuscript and the point-by-point response provided to the referee concerns that were raised during review at a different journal. In addition, and as mentioned before, we decided to involve two arbitrating experts to evaluate the revised version of your work, with respect to technical robustness, conceptual advance and overall suitability of your work for publication in The EMBO Journal.

As you will see from the reports provided below, advisor #1 is broadly in favour of the work stating the interest and value of your in vivo results and s/he is supportive of publication at The EMBO Journal. Advisor #2 points to concerns regarding the depth of the analysis of BASCs provided and in addition points to a number of experiments to complement the work and better distinguish it from the related, recently published study.

We have discussed all those points carefully in the team and concluded that we are overall positive on the study, however agree with advisor #2 that a more detailed characterization of the BASCs by single-cell RNAseq (arb adv # 2, pt. 1) would be required to make this study amenable for The EMBO Journal at this stage. In addition, we note that while per se not mentioned in the first set of reports, characterizing surface markers as Sca-1 on those cells should also help to better describe the phenotype and put it in the context of a controversial debate in the field (arb adv # 2, pt. 4). We expect these FACS experiments to be realistic for a minor revision.

Based on the overall positive experts' view together with our own assessment, we decided to proceed with publication of your work at The EMBO Journal pending the above points related to the advisor's input could be conclusively addressed in a time frame of two weeks.

Please note that while per se well taken, the arbitrator's points on human conservedness and additional organoid work (arb adv # 2, pts. 2, 4), are - as only brought up during arbitration - not relevant to the current study in our view and do not need to be addressed for publication at The EMBO Journal.

Once we have received the revised version, we should then be able to swiftly proceed with formal acceptance and expedited production of the manuscript

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REFEREE REPORTS:

Arbitrating advisor #1's comments:

This sounds to me like the authors ran into reviewers having strong opinions and leaving little room for their colleagues in this field. I like the paper and agree with your evaluation of it. The BASCs have remained rather controversial after Carla Kim and Tyler published their Cell paper. The current paper is of high quality and sheds light on this. I feel it can be published as is.

Arbitrating advisor #2's comments:

They need to respond to each reviewer point. They did not address many of the questions.

1) I do think that they will need to include the single cell RNA-seq to make it comparable to the other paper. The other paper did not do the best comparison which these authors have the opportunity to do: cherry+ vs gfp+ vs cherry+gfp+ . The other group isolated cells from different mice; the Braun group can isolate all populations from same mice.

2) Also if they see a unique signature of the BASCs, then they could compare to human single cell data to see if there is evidence for a human BASC. For example, is there a set of genes that define BASCs other than CCSP and SPC that might translate to human?
That has been a big question in the field. They should be able to do this fairly quickly with bioinformatics.

3) They should show if the Cherry+ GFP+ BASCs are Sca1-positive by FACS? Characterize for the markers EpCAM, Sca1, CD24.

4) The manuscript would benefit from data showing organoid culturing of the double positive cells.

Authors' correspondence 1st Apr 2019

We appreciate the helpful comments by the advisors. It should be no problem to provide FACS data about Sca1 expression in BASC as requested by advisor #2. Yet, I got a question, if I may, regarding the request for single cell RNA-seq (scRNA-seq). As you know, the sequencing depth provided by scRNA-seq is rather limited, essentially covering only around 15% of transcripts. Hence, we mostly focused on bulk RNA seq of isolated BASC, AT2 and Club cells, which allows a much better assessment of differences and similarities between BASC, AT2 and Club cells. We clearly see (using bulk RNA-seq) that BASCs are much more similar to AT2 than to Club cells but represent a different entity as indicated by PCA plots and Spearman correlation analysis. Furthermore, we identified a group of genes that is specifically enriched in BASCs compared to AT2 and Club cells.

Hence, I would feel much more comfortable to use the bulk RNA-seq rather than the scRNA-seq data.

Editors' Comments

We have discussed all those points carefully in the team and concluded that we are overall positive on the study, however agree with advisor #2 that a more detailed characterization of the BASCs by single-cell RNAseq (arb adv # 2, pt. 1) would be required to make this study amenable for The EMBO Journal at this stage.

Response: We now provided RNA-seq analysis of BASC, AT2, and Club cells in the revised manuscript, which reveal that BASCs share many features with Club cells and even more with AT2 cells. However, the RNA-seq analysis also reveals that BASCs cluster separately from AT2 and Club cells and differ in the expression of numerous genes.

In addition, we note that while per se not mentioned in the first set of reports, characterizing surface markers as Sca-1 on those cells should also help to better describe the phenotype and put it in the context of a controversial debate in the field (arb adv # 2, pt. 4). We expect these FACS experiments to be realistic for a minor revision.

Response: We have included a new figure describing FACS experiments, which demonstrate that CCSP+/SPC+ BASCs rarely express Sca1.

Please note that while per se well taken, the arbitrator's points on human conservedness and additional organoid work (arb adv # 2, pts. 2, 4), are - as only brought up during arbitration - not relevant to the current study in our view and do not need to be addressed for publication at The EMBO Journal.

Response: We appreciate the comments by arbitrating advisor #2. The detection of BASCs in humans and work with organoids is in our opinion clearly outside the scope of the current manuscript. We will address these questions in future studies.

The authors performed all requested editorial changes.

Arbitrating advisor #1's comments:

This sounds to me like the authors ran into reviewers having strong opinions and leaving little room for their colleagues in this field. I like the paper and agree with your evaluation of it. The BASCs have remained rather controversial after Carla Kim and Tyler published their Cell paper. The current paper is of high quality and sheds light on this. I feel it can be published as is.

Response: We thank the advisor for the encouraging comments.

Arbitrating advisor #2's comments:

1) I do think that they will need to include the single cell RNA-seq to make it comparable to the other paper. The other paper did not do the best comparison which these authors have the opportunity to do: cherry+ vs gfp+ vs cherry+gfp+ . The other group isolated cells from different mice; the Braun group can isolate all populations from same mice.

Response: We now provide RNA-seq analysis of BASC, AT2, and Club cells in the revised manuscript, which reveal that BASCs share many features with Club cells and even more with AT2 cells. However, the RNA-seq analysis also reveals that BASCs cluster separately from AT2 and Club cells and differ in the expression of numerous genes.

2) Also if they see a unique signature of the BASCs, then they could compare to human single cell data to see if there is evidence for a human BASC. For example, is there a set of genes that

define BASCs other than CCSP and SPC that might translate to human? That has been a big question in the field. They should be able to do this fairly quickly with bioinformatics.

Response: We thank the advisor for this comment. However, we sincerely believe that studies using human material is outside the scope of the manuscript. The RNAseq analysis revealed a number of putative markers that might be suitable for identification of human BASCs. Yet, selection and testing of fitting antibodies or probes for RNA FISH will require substantial time and effort with no guarantee of a positive outcome.

3) They should show if the Cherry+ GFP+ BASCs are Sca1-positive by FACS? Characterize for the markers EpCAM, Sca1, CD24.

Response: We have included a new figure describing FACS experiments, which demonstrate that CCSP+/SPC+ BASCs rarely express Sca1.

4) The manuscript would benefit from data showing organoid culturing of the double positive cells.

Response: We thank the advisor for this helpful suggestion. We agree that organoid cultures of double positive BASCs will be helpful to further demonstrate the ability of BASCs for generation of AT2 and Club cells. However, the current study focuses on the characterization of BASCs in vivo. A comprehensive characterization of the behavior of BASCs in organoid cultures is on its way and subject to a separate manuscript.

2nd Editorial Decision 10th Apr 2019

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Thomas Braun
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-102099

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	See below
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to predetermine sample size. For in vivo experiments including mouse strains with complex genotypes, a minimum of 3 up to 10 animals per group and time point were analyzed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Quantification of lineage-traced mCherry+ Club cells isolated by flow cytometry (Fig. 8B): one animal was excluded due to non-responsiveness in the naphthalene treatment group (data still representing a sample-size of n=5).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Since animals with different genotypes were subjected to treatment regimens, no randomization was possible
For animal studies, include a statement about randomization even if no randomization was used.	Mice of the same age (mostly littermates) were assigned into different cohorts based on the respective genotype, aiming at a balanced gender distribution between treatment groups and time points.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No, see below.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding to experimental conditions was not possible due to the apparent sickness of animals in response to epithelial injury. Blinding to genotypes in treatment groups was not possible since the investigator taking care of the mouse colony was also responsible for the entire downstream procedures including data analysis. However, due to consistent results across multiple biological replicates the authors believe that blinding was not relevant for the key experimental findings presented in this study.
5. For every figure, are statistical tests justified as appropriate?	Yes, for data comparison the non-parametric Mann-Whitney test was used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA, a non-parametric test was used
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Western blotting: mouse anti-FLAG® M2 (F1804, Sigma-Aldrich), rabbit anti-GFP (ab6556, Abcam). IHC: goat anti-CC10 (T-18, sc-9772, Santa Cruz Biotechnology), rabbit anti-proSPC (AB3786, Chemicon International), mouse anti-acetylated tubulin (T7451, Sigma-Aldrich). Flow cytometry: rat anti-Ly-6A/E (Sca-1) PE-Cy7, clone D7 (25-5981-81, eBioscience), rat IgG2a kappa isotype control PE-Cy7 (25-4321-81, eBioscience), rat anti-CD31 APC (17-0311-80, eBioscience), rat anti-CD45 APC (17-0451-82, eBioscience). All antibodies used are commercially available and are validated by the vendor for the assay and species used in this study.

USEFUL LINKS FOR COMPLETING THIS FORM

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http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
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http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jji.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cells (ATCC CRL-11268), murine v6.5 embryonic stem cells (C57BL/6x129/SV hybrid background). Cell lines were authenticated by morphology (microscopy). All cell lines are regularly tested for mycoplasma contamination (LookOut® Mycoplasma PCR Detection Kit, Company: Sigma-Aldrich) every 6 months.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	To generate chimeric mice, ES cells were expanded and injected into E3.5 donor blastocysts (isolated from 3 weeks old WT C57BL/6J females), which were subsequently implanted in pseudo pregnant recipients (7-15 weeks old NMRI females). To delete the neomycin selection cassette, adult (> 8 weeks) chimeric males were crossed to adult hACTB-FLPe deleter female mice (Tg(ACTFLPe)9205Dym). Cre- and tTA responder strains used in this study: Gt(ROSA)26Sor alias "Rosa26stopflox-lacZ", Gt(ROSA)26Sortm1(DTA)Lky alias "Rosa26stopflox-DTA", Tg(tetO-GFP,-lacZ)G3Rsp alias "tetObi lacZ/huGFP", Tg(tetO-cre)LC1Bjd alias "tetObi luc/Cre". For comparison of split-Cre/split-tTA mouse models, 3 months old BASC tracer and BASC viewer mice of both sexes were used. For lung injury models (naphthalene, bleomycin, IV infections), 8-12 weeks old BASC-specific knock-in strains (view, v-race, v-race DTA, both male and female) were used. To assess BASC contribution during homeostasis, 3 months old BASC v-race mice of both sexes were used. To analyze BASCs and descendants during embryonic development and ageing, E18.5, P6, 1 year and 2 year old BASC v-race mice of both sexes were used. For dox-dependent reversible BASC labeling, 3-4 months old BASC viewer mice of both sexes were used. To quantify the efficiency of BASC ablation, 3 months old BASC v-race and BASC v-race DTA mice of both sexes were used. To assess fluorescence expression patterns and functional inactivity of individual split-effector alleles, adult mice of both sexes were used. For comparative transcriptional profiling, BASCs, AT2 and Club cells were isolated by FACS from 8 weeks old BASC-specific knock-in strains of both sexes. Flow cytometric analyses to assess the Sca-1 status were performed on adult BASC viewer mice of both sexes. All animals were housed in individual ventilated caging (IVC) systems with food and water provided ad libitum on a 12 hour-based light/dark cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the local authorities.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance with respective guidelines is confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNAseq data have been deposited in the GEO database under GSE129440.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	RNAseq data have been deposited. Other data sets are provided as supplementary documents (source data).
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No