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GPNMB+Gal-3+ Hepatic Parenchymal Cells Promote Immunosuppression and Hepatocellular Carcinogenesis

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Transaction Report:

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr Wei,

Thank you again for the submission of your manuscript (EMBOJ-2023-114060) to The EMBO Journal, as well as for your patience with our feedback at this time of the year. As mentioned, your manuscript has been sent to three referees with expertise in liver biology and cancer, and we have received reports from two of them which are enclosed below. However, the third referee got delayed and did not provide his/her report even after repeated chasers; we have now decided to conclude on this based on the two existing reports.

As you will see from their comments, the referees acknowledge the potential interest and value of your findings. However, they also express major concerns, which need to be fully addressed before they can be supportive of publication of this work at the EMBO Journal. In more detail, both referees state that the claims on an in vivo functional role of GPNMB+, Gal-3+ hepatic parenchymal cells are not sufficiently support by the current data (ref#1, pts. 1,2; Ref#2, pt.1). Referee #1 also points to unclear relevance and dynamics of SOX18 as a driver of transformation (ref#1, pt.3).

Given the overall interest stated and broader angle of your findings, we can invite you to revise your manuscript experimentally to address the referees' comments. I need to stress though that we do require strong support from the referees on a revised version of the study to move on to publication of the work.

In light of the extensive experimentation requested, I would appreciate if you could contact me during the next weeks for exchange e.g. a video call to discuss your perspective on the comments and potential plan for revisions.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When submitting your revised manuscript, please carefully review the instructions below.

Please feel free to approach me any time should you have additional questions related to this.

Thank you for the opportunity to consider your work for publication.

I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised

manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) At EMBO Press we ask authors to provide source data for the main and EV figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (13th Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

Dr. Wei and colleagues reported a comprehensive single cell RNAseq profiling using a DEN induced HCC model in rat, and compared with human HCC patient single cell data to understand the potential cell types and mechanisms underlying HCC origin and malignant cell transformation. The authors identified an immunosuppressive subset of hepatic parenchymal cells marked by GPNMB+ and Gal-3+ in both rat model and human HCCs and claimed their arise from EPCAM+ population and as a major cell type contributing to the immunosuppressive microenvironment as well as HCC malignance. Mechanistically, the authors tried to identify key drivers that involved in the malignance transformation and SOX18 served as a candidate linking the expression of immunosuppressive genes GPNMB and Gal-1, HCC recurrence and poor prognosis. The good coverage of the single cell data from rat and human allows the detailed subset population analysis and the bioinformatic part of the study seems solid. However, the computation prediction driven conclusions need to be verified by experiments, especially in vivo. There are a few concerns that some of the conclusions from data profiling are not directly supported by the designed experiments.

Here are my major concerns:

1. By profiling the single cell seq during the progression of HCC in the DEN-rat model and patient HCC samples, authors identified the three major clusters of parenchymal cells: hepatocyte fraction(HCMeta), EPCAM+ cluster with differentiation potential(EP+Diff) and the mixed population of malignant cells and EP+Diff with an immunosuppressive signature(MTImmu). The increased percentage of the MTImmu population during time course as well as the velocity assay will not lead to the direct conclusion of EPCAM+ cells to MTImmu population transformation. For example, the clonal expansion and selection during tumorigenesis may contribute to the MTImmu population and the dynamic change of subset percentage may due to cell survival/fitness. The authors need to check this either by direct cell tracing or systematic study of the cellular status by histology/single cell gene expression.
2. Authors performed the EPCAM+ cell isolation from E13.5 liver followed by i.v. injection to study the process of EPCAM+ cell to HCMeta and MTImmu subsets transition. I have concerns about the relevance of this study to the hypothesis as the EPCAM+ hepatoblast cells from fetal liver are significant different as the native EPCAM+ liver cells during tumor formation although both are EPCAM+. It is not surprising that EPCAM+ hepatoblast cells are capable of differentiate to both hepatocytes and other subsets, and it is not clear if the EPCAM+GFP+ cell derived MTImmu subsets are direct transformation through the hepatocyte state. Manipulating the EPCAM+ cell directly in vivo may be challenging but the proper co-staining of EPCAM+ cells with the according subset markers during the time course need to be performed. Under healthy and injuries conditions, EPCAM+ cells in liver are mostly biliary epithelial cells and bipotential projectors are identified recently when all hepatocytes lost the capacity of proliferation by Bin Zhou lab. Authors need to show the kinetics of the EPCAM+ cells and their location in the tissue.
3. Authors showed the SOX18 as a key driver during the malignance transformation and immunosuppressive process by knockout down SOX18 in injected EPCAM+ cells. It would be more convincing if SOX18 are knockout in vivo directly without extra EPCAM+ cell administration as the role of SOX18 may involve in hepatoblast differentiation. Specifically, knockout SOX18 during different time window upon tumor formation will answer the question of the contribution of SOX18 during tumor initiation, progression or transformation.
4. SOX18 knockout and rescue experiment need to be performed to verify the interaction of immune cells with epithelial cells and the immunosuppressive effect.

A few minus points:
1. Figure 4K, 5L and 6F are too small and the IF staining is too weak to be evaluate the expression and localization of the markers.
2. Method section need more details about the experiment description. The EPCAM+ injection part is missing, and the detail of mice studies are needed.
3. The author identified 3 instead of 5 new TFs that may contribute to the malignance transformation

Referee #2:

The article by Meng et al. entitled "GPNMB+Gal-3+ Hepatic Parenchymal Cells Promote Immunosuppression and Hepatocellular Carcinogenesis" leverages single-cell sequencing in a DEN-induced liver cancer model and patients with HCC to identify changes in cell state during carcinogenesis. Initially, the authors identify 3 distinct populations of livers cells; namely HCmeta, which are characterized by HNF4a-driven expression of housekeeping metabolism genes; EP+Diff, which appear to be less

differentiated; and MTImmun, express genes relating to immunosuppression. Integrated analysis of the single cell datasets suggests that MTImmun are derived from EP+Diff, and that they transform into malignant cells. The authors then provide data suggesting that GPNMB+Gal-3+ MTImmu cells are tumorigenic and suppress CD8+ T cell immunity. Finally, the authors show that Sox18 is required for the transforming and immunosuppressive capabilities of GPNMB+Gal-3+ MTImmu cells. Overall, this manuscript is thought-provoking, however, some of the conclusions made are not well supported by the experimental data. In my opinion, there are some issues that should be addressed before the article could be considered for publication in the EMBO Journal.

Major Issues
1) In Fig 4I, GPNMB and Gal-3 as associated with stemness, but are they required for it? A knockdown study should be performed to test whether these are markers or genes necessary for these phenotypes.
2) In Fig 6E, I am confused as to how the authors achieved constitutive knockdown 16 weeks after injection of cells transiently transfected with siRNA targeting Sox18? Is this a loss of fitness phenotype?
3) In 6F, its not clear what you mean by a "physical juxtaposition of SOX18 with Gal-3
and GPNMB was observed in GFP+-derived cells in tumor". Wouldn't you be expecting these factors to act cell autonomously given the single cell analysis in 6A/B? It would be good to show higher mag images wherein Sox18 expression could be examined with GPMNB/Gal-3 in the presence of Hepatocyte and endothelial markers. Sox18 has been shown to be highly expressed in endothelial cells in numerous single cell datasets and it would therefore be good to exclude Sox18 positivity associated with vessels.
4) Fig 7C, E are difficult to see at this size. Show higher magnification of the IF-stained tissue. I also have a similar concern regarding the use of the term physical juxtaposition between GPMNB and Sox18. Are the authors suggesting that these interactions are non-cell autonomous?

Minor Issues
1) In Fig 6, is Sox18 expression sufficient to induce GPMNB and Gal-3? Similarly is Yap required for GPNMB and Gal-3 expression? It would be helpful to discern some of the mechanistic links.

Non-essential suggestions

1) Fig 1, I wonder how much the differences observed between the HCmeta and EP+Diff clusters is simply hepatocyte differentiation. The description of HCmeta seems to describe a normal hepatocyte, similarly, is the EP+Diff simply a dedifferentiated hepatocyte?
2) The use of RNA velocity in Fig 3 to define lineage is premature without subsequent validation studies. For example, I don't think you have data to claim, "MTImmu is derived from EP+Diff and can directly transform into malignant cells". Perhaps consider reiterating the caveats of this analysis.

Point-by-Point Response to Reviewers' Comments

Referee #1:

Dr. Wei and colleagues reported a comprehensive single cell RNAseq profiling using a DEN induced HCC model in rat, and compared with human HCC patient single cell data to understand the potential cell types and mechanisms underlying HCC origin and malignant cell transformation. The authors identified an immunosuppressive subset of hepatic parenchymal cells marked by GPNMB+ and Gal-3+ in both rat model and human HCCs and claimed their arise from EPCAM+ population and as a major cell type contributing to the immunosuppressive microenvironment as well as HCC malignance. Mechanistically, the authors tried to identify key drivers that involved in the malignance transformation andSOX18 served as a candidate linking the expression of immunosuppressive genes GPNMB and Gal-1, HCC recurrence and poor prognosis. The good coverage of the single cell data from rat and human allows the detailed subset population analysis and the bioinformatic part of the study seems solid. However, the computation prediction driven conclusions need to be verified by experiments, especially in vivo. There are a few concerns that some of the conclusions from data profiling are not directly supported by the designed experiments.

We thank the reviewer's constructive comments. We also understand the reviewer's concern about the verification part. To strengthen our study, we performed additional experiments to address each concern raised by this reviewer.

Major concerns:

1. By profiling the single cell seq during the progression of HCC in the DEN-rat model and patient HCC samples, authors identified the three major clusters of parenchymal cells: hepatocyte fraction (HCMeta), EPCAM+ cluster with differentiation potential (EP+Diff) and the mixed population of malignant cells and EP+Diff with an immunosuppressive signature (MTImmu). The increased percentage of the MTImmu population during time course as well as the velocity assay will not lead to the direct conclusion of EPCAM+ cells to MTImmu population transformation. For example, the clonal expansion and selection during tumorigenesis may contribute to the MTImmu population and the dynamic change of subset percentage may due to cell survival/fitness. The authors need to check this either by direct cell tracing or systematic study of the cellular status by histology/single cell gene expression.

Good point! It is difficult to describe the transformation from $EPCAM^{+}$ cells to the MT^{Immu} population only using the proportions of cell subpopulations. To address this issue, we sorted primary $EPCAM^{+}$ cells from GFP-labeled fetal livers (13.5 days) and injected them

into male rats pretreated with DEN for 3-4 weeks. The recipient rats were subjected to DEN treatment for 16 weeks (**Fig. R1 A**). Subsequently, we traced GFP-labeled EPCAM^{+} cells in the recipient rats at time points of 8- and 16- weeks post DEN-treatment. Supporting our original hypothesis, Gal-3^{+}GPNMB^{+} (MT^{Immu}) and AFP^{+} (malignance) cells were found to account for 28.7% and 14.2% of GFP-positive cells at 16 weeks post DEN-treatment, respectively (**Fig. R1 B**). Consistent with these results, immunofluorescence staining confirmed the co-staining of GFP with Gal-3, GPNMB and AFP in tumors of the recipient rats (**Fig. R1 C**). Together, these data corroborated the possibility of malignant transformation of EPCAM^{+} cells to GPNMB^{+}Gal-3^{+} MT^{Immu} cells.

To further examine the cellular status transition, we systematically analyzed the upregulated genes during the transformation from EP^{+Diff} to GPNMB^{+}Gal-3^{+} MT^{Immu} (**Figs. R1 D and E**). By coloring cells along the pseudotime line, we found EP^{+Diff} were mainly located at the beginning zone of the trajectory tree (branch #1), which directly transformed into MT^{Immu} (branch #3) (**Fig R1 D**). Functional clustering analysis of the upregulated genes during this conversion enriched typical characteristics of malignant tumors, such as negative regulation of immune response, positive regulation of EMT, and stem cell maintenance (**Figs. R1 E and F**). Therefore, these data indicate a malignant state of GPNMB^{+}Gal-3^{+} MT^{Immu} cells that most likely originated from EPCAM^{+} cells. We now include these results in the revised manuscript (updated **Figs. S4 B and E, 4 A and S5 E and F**).

Figure for reviewers removed

relevance of this study to the hypothesis as the EPCAM+ hepatoblast cells from fetal liver are significant different as the native EPCAM+ liver cells during tumor formation although both are EPCAM+. It is not surprising that EPCAM+ hepatoblast cells are capable of differentiate to both hepatocytes and other subsets, and it is not clear if the EPCAM+GFP+ cell derived MTImmu subsets are direct transformation through the hepatocyte state. Manipulating the EPCAM+ cell directly in vivo may be challenging but the proper co-staining of EPCAM+ cells with the according subset markers during the time course need to be performed. Under healthy and injuries conditions, EPCAM+ cells in live rare mostly biliary epithelial cells and bipotential projectors are identified recently when all hepatocytes lost the capacity of proliferation by Bin Zhou lab. Authors need to show the kinetics of the EPCAM+ cells and their location in the tissue.

Thanks for pointing out this issue. To address these questions, we performed multicolor IHC staining of MT^{Immu} markers (Gal-3, GPNMB) with endogenous $EPCAM^+$ cells in rats at 8- and 16- weeks after DEN treatment. Endogenous $EPCAM^+$ cells were located predominantly in the bile duct reaction area, consistent with previous studies (1, 2), under the injury conditions after 8 weeks exposed to DEN. At this point, $EPCAM^+$ cells barely expressed GPNMB or Gal-3 (**Fig. R2**). Interestingly, the co-staining of endogenous $EPCAM^+$ cells with GPNMB and Gal-3 were present in the tumor at 16 weeks after DEN treatment (**Fig. R2**). These results suggest that long-term chronic injury caused expansion and malignant transformation of endogenous $EPCAM^+$ cells, consistent with our and other previous studies (3-5).

Figure for reviewers removed

3. Authors showed the SOX18 as a key driver during the malignance transformation and immunosuppressive process by knockout down SOX18 in injected EPCAM+ cells. It would be more convincing if SOX18 are knockout in vivo directly without extra EPCAM+ cell administration as the role of SOX18 may involve in hepatoblast differentiation. Specifically, knockout SOX18 during different time window upon tumor formation will answer the question of the contribution of SOX18 during tumor initiation, progression or transformation.

The reviewer raised an interesting and important question! We totally agree that depletion of SOX18 would be ideal in terms of confirming the contribution of SOX18 during tumor initiation and development. Given the difficulties in efficiently depleting SOX18 in DEN-induced HCC rat model within 3 months, we inhibited SOX18 via Sm4 (a known SOX18 small-molecule inhibitor) (6) starting from 8- or 12- weeks to 16 weeks after DEN treatment. Of note, to dynamically evaluate the effects of SOX18 on tumor initiation and progression, we collected liver tissues at 12- and 16- weeks after DEN treatment (**Fig. R3 A**). We found rats treated with Sm4 starting at 8 weeks had significantly fewer preneoplastic lesions than the mock group, which displayed several small, white, cyst-like lesions, at 12-week after DEN exposure, indicating that the inhibition of SOX18 dramatically suppressed the initiation of HCC (**Figs. R3 B and C**). Rats treated with Sm4 starting from 12 weeks did not develop HCC as severe as those observed in the mock group, suggesting that inhibition of SOX18 significantly suppressed HCC progression (**Figs. R3 B and C**).

Moreover, inhibition of SOX18 starting from 8 weeks significantly attenuated the expansion of endogenous $EPCAM^+$ cells and their co-staining with Gal-3, GPNMB and AFP in rats exposed to DEN after 16 weeks (**Figs. R3 D and E**). Inhibition of SOX18 starting at 12 weeks did not remarkably repress the expansion of endogenous $EPCAM^+$ cells, but notably reduced the co-expression of $EPCAM^+$ cells with Gal-3, GPNMB and AFP in rats exposed to DEN after 16 weeks (**Figs. R3 D and E**). The results verified the essential role of SOX18 during tumor initiation and progression, as well as the transformation of $EPCAM^+$ cells. We now include these results in the revised manuscript (updated Figs. **S8 A-E**).

Figure for reviewers removed

4. SOX18 knockout and rescue experiment need to be performed to verify the interaction of immune cells with epithelial cells and the immunosuppressive effect.

Following the reviewer's suggestion, we performed SOX18 knockdown and rescue experiments in GPNMB$^+$Gal-3$^+$ cells, and co-cultured them with blood-derived CD45$^+$CD3$^+$ T cells. Indeed, co-culture with SOX18-knockdown GPNMB$^+$Gal-3$^+$ markedly enhanced the percentage of CD8$^+$granzyme B (GZMB)$^+$ T cells (6.22% vs. 13.7%), while decreased the fraction of exhaustive CD8$^+$PD-1$^+$ T cells (9.24% vs. 6.41%). Notably, re-introduction of SOX18 in GPNMB$^+$Gal-3$^+$ cells dramatically decreased CD8$^+$GZMB$^+$ T cells to 9.13%, and efficiently rescued the exhausted CD8$^+$PD-1$^+$ T cells to 15.6% (**Fig. R4 A and B**). Together, these results again confirmed the key role of SOX18 in the immunosuppressive GPNMB$^+$Gal-3$^+$ cells. We now include these results in the revised manuscript (updated **Figs. 6 F and G**).

Figure for reviewers removed

Minus points:

5. Figure 4K, 5L and 6F are too small and the IF staining is too weak to be evaluate the expression and localization of the markers.

Thanks for pointing out this issue. We now enlarge the size of the IF images and increase the staining resolution to ensure a clearer assessment of the expression and localization of markers (updated **Figs. 4 I, 5 L and 6J**).

Figure for reviewers removed

6. Method section need more details about the experiment description. The EPCAM+ injection part is missing, and the detail of mice studies are needed.

Thank you for pointing this out. We improved the Method section. In particular, we completed the description of $EPCAM^+$ cell injection in the section "MATERIALS AND METHODS". Briefly, we removed fetal liver tissues from GFP-labeled rats at day 13.5 of gestation. And then fetal liver tissue was dissociated to obtain cell suspension after enzymatic digestion for 10 min at 37°C. $EpCAM^+$ cells were isolated with immunomagnetic beads (**Fig. R6 A**). 1×10^8 GFP-labeled $EPCAM^+$ cells were injected via tail vein into DEN-treated male rats (**Fig. R6 B**, red arrows). The injection started from 3-4 weeks after DEN induction for 3 times, once a week. Rats were continually subjected to DEN treatment for 16 weeks, and sacrificed at 4, 8, 12 and 16 weeks for sample collection (black arrows). 0.9% NaCl was used in the mock group (**Fig. R6 B**).

Figure for reviewers removed

7. The author identified 3 instead of 5 new TFs that may contribute to the malignance transformation.

Many thanks. We corrected it in the revised manuscript.

Referee #2:

The article by Meng et al. entitled "GPNMB+Gal-3+ Hepatic Parenchymal Cells Promote Immunosuppression and Hepatocellular Carcinogenesis" leverages single-cell sequencing in a DEN-induced liver cancer model and patients with HCC to identify changes in cell state during carcinogenesis. Initially, the authors identify 3 distinct populations of livers cells; namely HCmeta, which are characterized by HNF4a-driven expression of housekeeping metabolism genes; EP+Diff, which appear to be less differentiated; and MTImmun, express genes relating to immunosuppression. Integrated analysis of the single cell datasets suggests that MTImmun are derived from EP+Diff, and that they transform into malignant cells. The authors then provide data suggesting that GPNMB+Gal-3+ MTImmu cells are tumorigenic and suppress CD8+ T cell immunity. Finally, the authors show that Sox18 is required for the transforming and immunosuppressive capabilities of GPNMB+Gal-3+ MTImmu cells. Overall, this manuscript is thought-provoking, however, some of the conclusions made are not well supported by the experimental data. In my opinion, there are some issues that should be addressed before the article could be considered for publication in the EMBO Journal.

We thank the reviewer for the encouraging and constructive comments.

Major Issues:

1. In Fig 4I, GPNMB and Gal-3 as associated with stemness, but are they required for it? A knockdown study should be performed to test whether these are markers or genes necessary for these phenotypes.

Thanks for pointing out this issue. To validate the roles of GPNMB and Gal-3 on stemness, we knocked down *GPNMB* or *Gal-3* and performed sphere formation assay to assess the self-renewal ability of GPNMB$^+$Gal-3$^+$ cells after 1 weeks. Consistent with our original results, knockdown of *Gal-3* and *GPNMB* in EPCAM$^+$ cells significantly reduced their sphere formation efficiencies (**Figs. R7 A and B**). Together, these data clearly indicate an essential role for Gal-3 and *GPNMB* in maintaining the stemness of GPNMB$^+$Gal-3$^+$ cells. We accordingly incorporated these results into the revised manuscript (updated **Figs. 4 G and H**).

Figure for reviewers removed

2. In Fig 6E, I am confused as to how the authors achieved constitutive knockdown 16 weeks after injection of cells transiently transfected with siRNA targeting Sox18? Is this a loss of fitness phenotype?

We are sorry for missing the description regarding *SOX18* knockdown by lentivirus. We now added the related description in the section "MATERIALS AND METHODS" of revised manuscript. Briefly, oligonucleotides with the following targeting sequences were used for the cloning of siRNA-encoding sequences in hU6-MCS-PGK-EGFP lentiviral RNAi vector (Hanbio, Shanghai, China): *SOX18*, 5'-GACCATCCCAACTACAAGT-3'. The recombinant lentivirus with targeting sequences of *SOX18* was produced by co-transfection of 293T cells with plasmids PSPAX2 and PMD2G with LipoFiter (Hanbio, Shanghai, China). Lentivirus-containing supernatant was then harvested 48 h after transfection and filtered through 0.22-μm cellulose acetate filters (Millipore, USA). Recombinant lentiviruses were concentrated by ultracentrifugation (2 h at 50,000×g).

Approximately 0.5–2 μg/ml recombinant lentiviruses were transfected according to the manufacturer's instructions. $EPCAM^+$ cells were infected with vector (MOI 10) or si*SOX18* (MOI 15) in serum-free medium for the indicated times. 8 h later, cells were rinsed and cultured in fresh medium. After 48h, cells were cultured in DMEM medium with 10% FBS. We found that most of the $EPCAM^+$ cells (>80%) expressed EGFP 48 hours after the transfection. The empty lentivector lenti-EGFP was used as a negative control. Three

days after viral infection, 10^6 cells were harvested and examined for the level of *SOX18* by Realtime-PCR.

3. In 6F, its not clear what you mean by a "physical juxtaposition of SOX18 with Gal-3 and GPNMB was observed in GFP+-derived cells in tumor". Wouldn't you be expecting these factors to act cell autonomously given the single cell analysis in 6A/B? It would be good to show higher mag images wherein Sox18 expression could be examined with GPNMB/Gal-3 in the presence of Hepatocyte and endothelial markers. Sox18 has been shown to be highly expressed in endothelial cells in numerous single cell datasets and it would therefore be good to exclude Sox18 positivity associated with vessels.

Sorry for the confusing presentation. The sentence "physical juxtaposition of SOX18 with Gal-3 and GPNMB was observed in GFP^+-derived cells in tumor" was meant for that the co-staining of SOX18 with Gal-3 and GPNMB was observed in GFP-labelled cells. We now increased the magnification of the IF images to ensure a clearer observation of the co-localization of SOX18 with Gal-3 and GPNMB. To exclude $SOX18^+$ vascular endothelial cells, we labelled $CD34^+$ cells in this immunofluorescence staining. The oval $Gal\text{-}3^+GPNMB^+SOX18^+$ cells (yellow arrow) were significantly different from that of flat $CD34^+SOX18^+$cells (red arrow), thus we preliminarily excluded the interference from $CD34^+SOX18^+$ endothelial cells. High expression of SOX18 was observed in $GPNMB^+Gal\text{-}3^+$ cells (yellow arrows) (**Fig. R8**).

Figure for reviewers removed

4. Fig 7C, E are difficult to see at this size. Show higher magnification of the IF-stained tissue. I also have a similar concern regarding the use of the term physical juxtaposition between GPNMB and Sox18. Are the authors suggesting that these interactions are non-cell autonomous?

Following the reviewer's comments, we improved the magnification of the IF images (**Fig. R9 A and B**). The wording "physical juxtaposition between GPNMB and Sox18" was meant for co-location of SOX18 with Gal-3 and GPNMB. We now replace "physical juxtaposition" with "co-expression of SOX18 with Gal-3 and GPNMB " in the revised manuscript.

Figure for reviewers removed

Minor Issues:

5. In Fig 6, is Sox18 expression sufficient to induce GPNMB and Gal-3? Similarly is Yap required for GPNMB and Gal-3 expression? It would be helpful to discern some of the mechanistic links.

Thank you for pointing this out. In original **Fig. S7 A**, we performed RT-PCR with or without knockdown of *Sox18*, *Tead2* or *Hdac2*. Interestingly, *Lgals3* was downregulated after knockdown of *Sox18* and *Tead2*, and *Gpnmb* was downregulated after knockdown

of *Hdac2* and *Sox18*, suggesting that Sox18 rather than Tead2/Yap was required for the expression of GPNMB and Gal-3 (**Fig. R10 A and B**).

Figure for reviewers removed

6. Fig 1, I wonder how much the differences observed between the HCmeta and EP+Diff clusters is simply hepatocyte differentiation. The description of HCmeta seems to describe a normal hepatocyte, similarly, is the EP+Diff simply a dedifferentiated hepatocyte?

This reviewer raised a very important question! To clarify the difference between the HC^{Meta} and EP^{+Diff}, we performed functional cluster analysis of differentially expressed genes. By coloring cells along the pseudotime, we found EP^{+Diff} were mainly located at the beginning zone of the trajectory tree (branch #1), which eventually evolved into HC^{Meta} in one branch (branch #2) (**Fig. R11 A**). Additionally, we extracted the upregulated genes in the conversion from branch #1 to branch #2 (**Fig. R11 B**). GO analysis enriched several biological processes associated with cholesterol metabolism and homeostasis, lipid biosynthesis and transport (**Fig. R11 C**). Importantly, this subpopulation highly expressed molecules related to liver metabolism, so we named them HC^{Meta}. Since we observed the characteristics of single cells at different time points during DEN-induced rat model, clusters 1-3 might contain the cells during the

differentiation from stem cell/precursor cells to hepatocytes, mature hepatocytes or proliferative hepatocytes induced by injury. Meanwhile, EP^{+Diff} (clusters 4-6) highly expressed differentiation-related markers including *Epcam*, *Cd44*, *Cd24* and *Aldh1a1* (7-9). Thus, EP^{+Diff} subpopulation could include stem/precursor cells and dedifferentiated hepatocytes, which were induced at the liver injury state and displayed differentiated potential (10).

Figure for reviewers removed

7. The use of RNA velocity in Fig 3 to define lineage is premature without subsequent validation studies. For example, I don't think you have data to claim, "MTImmu is derived from EP+Diff and can directly transform into malignant cells". Perhaps consider reiterating the caveats of this analysis.

As advised by the reviewer, we now revise our description of results as below.

Indeed, we performed RNA velocity analysis to examine the conversion direction and observed that the EP^{+Diff} population exhibited a directional flow towards MT^{Immu} and malignant cells. Together, these results indicated the potential transformation of EP^{+Diff} to MT^{Immu}. We now accordingly incorporate these descriptions into the revised manuscript.

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Dear Dr Wei,

Thank you for submitting your revised manuscript (EMBOJ-2023-114060R) to The EMBO Journal. As mentioned, your amended study was sent back to the referees for their re-evaluation, and we have received comments from both of them, which I enclose below.

As you will see, the experts stated that the work has been substantially improved by the revisions and they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining discussion point by referee #1 carefully and amend the text where appropriate.

Also, we now need you to take care of a number of minor issues related to formatting and data presentation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you might have noted on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
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The EMBO Journal

****

Formatting changes required for the revised version of the manuscript:

>> Adjust the title of the 'Declaration of Interests' section to 'Disclosure and Competing Interests Statement'.

>>Remove the 'Key Points' from the manuscript text.

>> Author Contributions: Please remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.
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>> Adjust the reference format to EMBO Journal style, limiting to 10 authors et al. .

>> Appendix file: amend the ToC on the first page with page numbers. Change the nomenclature to Appendix Figure S1-S8 and Appendix Table S1-S3 and adjust the corresponding callouts in the text.

>> Please provide Source Data compiled as one file per figure (compare attached source data request summary by my colleague H. Sonntag).

>> Introduce ORCID IDs for all corresponding authors (A.H., Y.M.) via our online manuscript system. Please see below for additional information.

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Thank you very much in advance.

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Referee #1:

I thank the authors to perform additional experiments to address all my concerns. With the revised version, I believe the story of single cell dynamics of tumor initiation is more round and solid.
I suggest to add a paragraph of study limitations in the discussion section regarding the modeling of tumor initiation and progression via the administration of EpCam+ cells. Except that, I don't have additional questions.

Referee #2:

The authors have provided thoughtful responses, which address the issues initially raised. I have no more concerns or queries. The new data provides even more compelling evidence of the importance of this pathway in HCC, which will be of broad interest in the hepatology and cancer fields.

The authors addressed the remaining editorial issues.

2nd Revision - Editorial Decision **4th Oct 2023**

Dear Dr Wei,

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.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figures included in this file.

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If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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The data shown in figures should satisfy the following conditions:

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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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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).
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