Manuscript EMBO-2014-89279

**Spontaneous development of hepatocellular carcinoma with cancer stem cell properties in PR-SET7-deficient livers**

Kostas C. Nikolaou, Panagiotis Moulos, George Chalepakis, Pantelis Hatzis, Hisanobu Oda, Danny Reinberg and Iannis Talianidis

*Corresponding author: Iannis Talianidis, Biomedical Sciences Research Center Alexander Fleming*

---

**Review timeline:**

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>16 June 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>18 July 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>24 September 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>30 October 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>05 November 2014</td>
</tr>
<tr>
<td>Accepted</td>
<td>11 November 2014</td>
</tr>
</tbody>
</table>

**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. The original formatting of letters and referee reports may not be reflected in this compilation.)

*Editor: Alexander Kohlmaier*

1st Editorial Decision 18 July 2014

Thank you for submitting your study "Oncogenic transformation of differentiating ductal progenitors gives rise to cancer stem cells" for consideration in the EMBO Journal. We have now received the full reports of four expert reviewers, which you will find copied below. As you will see, the referees' specific comments are well-considered and constructive. While all referees acknowledge that your findings are of significant interest in principle, all of them voice substantial concerns and considerations that question whether the cellular origin of the hepatocellular carcinomas can be derived from the current dataset.

Specifically, all four referees concur that decisive positive lineage tracing and additional co-labeling should be performed to be able to more conclusively determine whether hepatocytes escaping Cre-mediated recombination in the regenerative areas are the cells of origin for HCC onset in the PR-SET7 KO model. Several lines of evidence are offered by the referees to support this escaper-hypothesis. As such, whether these cells or biliary ductal progenitors undergo oncogenic transformation during onset of HCC in your model remains to be determined in a candidate for publication in our journal.

Secondly, representing the core topic of this manuscript, and pointed out most clearly by referee #4 (points 3/4), we were also left with the question whether cancer stem cells are developing at all in the PR-SET7 KO model. We think that it is, therefore, indeed critical to address whether the cancer
cells of origin in your model do self-renew while also giving rise to phenotypically distinct non-tumorigenic cells during HCC growth in the PR-SET7 KO.

Together, we would like to invite you to submit a revised version of your manuscript for evaluation to the EMBO Journal attending to these two major points. We do realize that the experimental revision requested is substantive. In case you feel that addressing these points is beyond the scope of your current project, we would also understand if you decided to submit your manuscript elsewhere. Nevertheless, we would encourage you to submit a revised manuscript to our journal. We generally allow three months as standard revision time, but in this case and if required we would certainly offer to extend the revision deadline. I also want to point out that it is our policy that competing manuscripts published during the agreed revision period would have no negative impact on our final assessment of your revised study.

Please do contact me by reply to this mail if you have questions regarding this decision. In the meantime, thank you for the opportunity to consider you work for the EMBO Journal. I am looking forward to hearing from you!

------------------------------------------------

REFEREE REPORTS

Referee #1:

The manuscript from Talianidis lab describes that the methyltransferase PR-SET7 in postnatal liver results in liver damage with subsequent induction of a ductal proliferative response. Prolonged damaged to the tissue increases STAT3 signaling and results in hepatocellular carcinoma originated from the ductal progenitors. While the finding that PR-SET7 is implicated in hepatocellular carcinoma via the induction of DNA damage to liver cells is a very interesting observation supported by the data, the origin of the cells that give rise to these tumors is not fully addressed. The authors use the presence or absence of mono or tri-methylation but markers for both lineages are missing. Would the authors be able to fully address these points (see below), this manuscript would be of interest to the liver community and the first report indicating the direct role of a methyltransferase in the development of liver cancer.

Comments:

1) PR-SET7 deletion in embryonic and adult liver:

The authors use a previously generated floxed allele PR-SET7loxp and crossed with several Cre lines. When PR-SET7 is deleted in the embryonic liver (by AlIfCre) the authors demonstrate that this enzyme is critical for normal liver organogenesis. This section is clear and supported by the data. Then the authors evaluate the effect of deleting PR SET7 in later stages. For that an AlbCre line is used. Albeit it is clear that this animals develop a normal liver and do not have the same phenotype of the AlIfCre, the authors state that this is a "adult" model. However, it is well known that this cre line is expressed in liver progenitors. The authors use the word "postnatal" but then consider this one adult liver. Actually the authors themselves show that Alb is expressed at E18.5 (Fig 1C). The authors use the word "postnatal" but then consider this one adult liver. This section needs rewriting to avoid confusion in that line.

2)- Loss of PR_SET7 induces DNA damage and necrosis in dividing cells.

The authors observe that there are 3 areas where liver cells are different: "healthy" (area A), enlarged /damaged (area B), regenerated (Area C).

This part of the data is not clear and lacks in depth analysis. According to the authors, the big cells are hepatocytes that are necrotic. They base this statement in 2 pieces of data: the absence of tunel positive cells (which according to them in Figure 2D correspond to infiltrating non-hepatic cell types) and the electronmicroscopy analysis presented in figure 3. However, There is no co-labeling that proves that the "enlarged" cells are or derived from hepatocytes. Markers of co-labeling are missing. Might be useful to the authors to stain the paraffin sections with Alb or cyp or hnf4a, which would facilitate the visualization of the different zones and
different sizes of cells. It is difficult to assess which area is which in the immunofluorescence presented.

On the same line, the authors assume that tunel positive cells are infiltrating cells. Again, colabeling is missing there to identify the tunel positive population.

What it can be conclude from the data presented it that the DNA damaged cells stained by gH2AX are alb+ cells, but that these cells are the "big" cells on area B, and that these were derived from damaged hepatocytes, is not clear from the data presented.

On another line, The big cells have lost the monomethyl mark in K20. Then the authors conclude that this is because they become tri-methylated. But, in figure 3B, every cell from area A, B or C are try-methylated, can the authors explain that?

Also, Do this areas A, b, C correspond to hepatocytes in zone 1, 2 or 3? (i.e. from Central vein-to portal region)

3) Loss of PR-SET7 induces repopulation of the liver from a ductal progenitor that undergoes oncogenic transformation
The authors observe a ductal /progenitor response following PR-SET7 deletion. That is an interesting observation clearly supported by the data. Then the authors mention that these ductal /progenitors undergo an oncogenic transformation. This assumption is based on p-STAT3 staining. But, is this activation of the STAT3 pathway occurring in the expanding ductal progenitors? Co-labeling is missing.

4) the tumors derived from the PRSET7 mutants display a cancer stem cell phenotype.
The authors perform a series of elegant transcriptomic analysis and conclude that the cells forming these tumors have a "Stem" cell phenotype.

However, the cell of origin of these "tumor stem cells" is not clear from the data. The authors state that tumors in PR-SET7 mutant mice arise form ductal /progenitor cells. However, one can envision the scenario that the cre is not 100% efficient so, the tumoral cells could be derived from hepatocytes where PR-SET7 is not deleted and that these repopulate the remaining liver while undergoing an oncogenic transformation. Could the authors quantify the extent of the Cre-deletion?

That last point would benefit/require lineage tracing experiment to fully rule out the cell of origin in these tumors. In the absence of this data, the authors should tone down the cell of origin of the tumor. That still doesn't hamper the conclusion of the paper, where SET7 is essential to maintain liver homeostasis and in its absence liver damage is induced, which can contribute to an oncogenic transformation of the tissue.

Referee #2:

Nikolaou and colleagues investigate the liver-specific deletion of PrSet7 in mouse. Deletion of PrSet7 during embryogenesis is lethal. Therefore Nikolaou et al. switch to Albumin-Cre mediated deletion of PrSet7 which only occurs in adult liver. They show that PrSet7 protein is lost 45 days after birth (P45). In P120 mice, PrSet7-deficient livers display signs of hepatocyte necrosis, inflammation and fibrosis. Later in life (P240), PrSet7 ko mice develop hepatocellular carcinomas. Appearance of ductal cell markers (A6, Sox9) in regenerating areas of PrSet7-deficient livers early in life and cell cycle arrest of PrSet7-deficient hepatocytes suggest that hepatocellular carcinomas in these mice develop from ductal progenitor cells and not from excessively proliferating hepatocytes. Thus the authors have generated a novel mouse model for hepatocellular carcinoma with unique characteristics which is potentially useful for translational studies.

A major point of the manuscript is that hepatocellular carcinomas in PrSET7 ko mice develop from ductal progenitor cells. The line or argumentation is that (a) PrSet7-deficient hepatocytes arrest in cell cycle when they start proliferation (b) ductal cell markers can be detected in regenerative zones...
of PrSet7 ko livers (c) gene expression profile between hepatocyte-derived HCC progenitors and PrSet7 ko livers is quite different. Although these points are highly suggestive for the ductal progenitor origin of PrSet7 HCCs; they are not entirely conclusive. Especially the point that deletion escaper cells could contribute to HCC cannot easily be excluded. It is clear that the majority of hepatocytes in P45 mice has lost PrSet7. Thus, severe damage, like a partial hepatectomy cannot be compensated by spurious deletion escapers. However, continued low-level damage over 240 days is a different scenario. In support of the deletion escaper hypothesis is actually the fact that in P240 livers PrSet7 is ubiquitously detectable in livers (Fig. E4B) although the cells express high levels of Albumin (Fig. 6D) whose promoter drives Cre expression and should lead to deletion of PrSet7. In other words, PrSet7 clearly escapes deletion in the regenerative areas. Otherwise regenerative processes and development of HCC would probably not be possible due to cell cycle arrest. Ultimately, lineage-tracing experiments are probably the only way to clearly demonstrate the origin of HCC in PrSet7 ko mice. In absence of a clear demonstration I would recommend to tone down the title of the manuscript and to modulate the discussion accordingly.

Additional points:

(1) The authors make a point that deletion of PrSet7 is already complete in P20 mice. I could not find data supporting this argument.

(2) The analysis of cell duplications in postnatal livers (Figure E1D) is not completely clear to me and needs to explained better. Total DNA in 0.1mg liver should be similar at P20 and P240 if cell density does not change. What is known about the proliferation of individual hepatocytes? Do all hepatocytes divide at some point during their life cycle?

(3) What is the penetrance of liver cancer in P240 mice?

(4) In arrested PrSet7 ko hepatocytes H4K20me1 is reduced and H4K20me3 is still present (Figure E2C). The authors argue that loss of H4K20me1 may be due to conversion to me3. How can this result be explained in the light of previous data that demonstrated loss of H4K20me3 upon PrSet7 deletion (e.g. Oda 2009, PMID:19223465; Driskell 2012, PMID:22117221)?

Referee #3:

First of all I think this is a definite contribution to the field. The experimental scenario is interesting and novel and the phenotype is strong and convincing. The authors first tested an AFP cre driven deletion of PR-SET7, which gave lethality and lack of hepatocyte growth, before adopting an albumin cre system. Here the PR-DET7 deficient hepatocytes die and a ductular response is seen with a FGF7 positivity. Hepatocellular carcinoma was seen in the in P240 PR-SET7ΔHepA mice. The resultant tumours are hepatocellular and indeed there is a strong ductal reaction that is associated with the development of hepatocellular carcinoma. Because PR-SET7 deletion causes G2 phase arrest and necrotic cell death in hepatocytes it is claimed that the HPCs (PR-SET7 intact- immune-positive for H4K20Me1 or PR-SET7) must therefore be the source of HCC. The claim is that hepatic progenitors give rise to hepatocellular carcinoma but this is not directly proven using a positive lineage tracing experiment. As such there is a residual concern that there could be an alternative explanation for the results i.e. that the HCCs are due to hepatocytes that were un-recombined by the albumin cre. This comes to my main issue - the title "Oncogenic transformation of differentiating ductal progenitors gives rise to cancer stem cells in hepatocellular carcinoma". My contention is that for such a strong and important claim to be made either a positive tracing technique should be employed, the claim should be modified or the authors should be able to argue or demonstrate that there is zero chance that the HCCs are not arising from an unrecombined hepatocyte that could then undergo ductular change and thereby express all the biliary/ductular markers mentioned in the text (e.g. Sox9, A6 etc., the CD133 staining here is an unusual pattern to me). Xenografts are performed and again suggest a ductular / progenitor phenotype as does a gene expression profile from the cancer bearing livers.
It is unclear to me whether this PR-SET7 deletion is relevant to human HCC and such human data would strengthen the paper.

Obviously many HCCs have progenitor phenotypes and a growing view is that this may represent phenotypic change rather than reflecting origin. Recent work has suggested using lineage tracing that hepatocytes undergo a ductular change during injury and this view should be taken into account and discussed.

Overall this is a clear paper that relies heavily on immunostaining and negative tracing of progenitors into HCC. How reliable this is depends upon the recombination efficiency.

Referee #4:

Nikolaou et al. use a novel carcinogenesis in which hepatic deletion of PR-SET7 induces cancer formation from a progenitor pool in the liver. Based on these findings, the authors conclude that ductal progenitors give rise to cancer stem cells.

This reviewer has reviewed the manuscript in a previous version for a different journal. As the manuscript has not been modified in major manner, many of the comments remain the same:

Major

1. The presented finding are interesting but there are concerns about relevance and interpretation of the data: (I) Is this a valid model for human HCC? The main mechanism of carcinogenesis is through an escape route after blocking the ability of normal hepatocyte to replicate. However, in human hepatocarcinogenesis differentiated hepatocytes typically exist side by side with progenitor-like cells, and it appears unlikely that such a scenario takes place. In fact, there is typically chronic hepatocyte regeneration/proliferation alongside expansion of proliferating progenitor cells - never a total block of hepatocyte proliferation. (II). Is there a known role for PR-SET7 in human hepatocarcinogenesis? The cited study by Takawa et al. shows increased expression of SETD8 whereas the authors delete PR-SET7. This is not further discussed and seems to be the opposite.

2. The authors need to include actual data how this model differs from mouse models of HCC in which deletion of key survival genes such as Tak1 or Nemo is ablated. Is a similar escape observed in these models and degree of CSC-like cells in these models or is the PR-SET7 model unique? The generation of a condition that does not allow hepatocytes to replicate is naturally going to promote expansion of populations that escape from this. There is simply no human condition that leads to such a drastic impairment of hepatocyte replication and such a profound selection mechanism. Of note, progenitors in human liver are likely to generate functional hepatocytes whereas progenitor cells in the PR-SET7 model will never give rise to mature cells as their offspring will die immediately. While the presented experimental findings are undoubtedly correct, the question whether the model has relevance for the human situation and can give clues to the origin of human HCC remains unanswered. It is hard to judge whether this is simply an exaggeration of the human situation, or whether it is simply an unphysiological model. Likewise, it should be considered that hepatocytes have the ability to de-differentiate into immature progenitor like cells via Hippo/Yap (Cell 2014).

3. The authors need to prove the presence of cancer stem cells in their model. The hierarchical model of cancer stem cells implies that some of cells in the tumor are true cancer stem cells with unlimited proliferation potential, but that they also give rise to a distinct set of cells that are non-tumorigenic. Hence, the authors need to (I) prove the presence of these two cell populations, and (II) show that upon transplantation, purified tumorigenic “cancer stem cells” give rise to more tumorigenic cells as well as phenotypically distinct nontumorigenic cells. This clarification is not only important for proper nomenclature but to determine whether this is truly hierarchially organized tumor (as in HCC) or simply an accumulation of progenitors/stem cells because the extremely high selective pressure that will more or less kill any differentiated hepatic cell.

4. In view of the above questions, there needs to be a much more thorough characterization of tumors. Many of the PCR markers are progenitor rather than HCC markers. Are these true HCCs? The degree of immature cells suggest that these cells may be different from most HCCs.
Comparison of gene expression profiles to human HCC, cholangiocarcinoma (the PR-SET 7 could also be a form of intrahepatic cholangiocarcinoma given its proposed cellular origin) and mixed HCC-CC should be added. In addition, more careful characterization of tumors should be performed including collagen IV staining, cytokeratin 19 staining and genomic analysis such as CGH (to understand whether this is a pure expansion of immature cells - or true cancer driven by specific genomic events). The authors should also determine whether there is metastasis to the lung, as commonly observed in late stage HCC.

5. What is the penetrance in this model? The majority of patients with chronic liver disease and cirrhosis do not develop HCC. If 100% of mice develop HCC, then the model does not reflect the pathogenesis of human HCC. A more valid model might be ablation of PR-SET7 in a subset of hepatocytes, e.g. through a less efficient Cre approach (e.g. Alb-CreERT). Have the authors tested if this be sufficient to trigger cancer? Such a model would also be more physiological than in utero deletion (occurring in most Alb-Cre hepatocytes).

6. The discussion is too lengthy.

Minor

1. It is not clear why the authors use immunosuppressed mice for their transplant models. Hepa1-6 cells can be easily engrafted into syngenic mice. The same should be true for PR-SET7 cells. The authors should try this approach.

2. The authors state "postnatal deletion" or "heaptocyte-specific deletion" using the albumin-Cre mice. Deletion occurs in utero and also includes the biliary compartment (possibly progenitors). Hence, the terminology should be altered.

Response to the reviewers

We are pleased that the reviewers found that our paper is of significant interest to the field. We would like to thank them for the constructive comments and specific suggestions, which improved its quality. We agree with the comments and performed most of the suggested experiments and included the requested modifications in the text.

Our response addressing the concerns of the reviewers is divided into two parts: First, we provide our answers for the 2 main issues that have been raised by most reviewers and then continue by answering point-by-point the comments.

Main Issue 1: Origin of HCC in PR-SET7 KO model

Referee 1 point 4: the tumors derived from the PRSET7 mutants display a cancer stem cell phenotype. The authors perform a series of elegant transcriptomic analysis and conclude that the cells forming these tumors have a "Stem" cell phenotype. However, the cell of origin of these "tumor stem cells" is not clear from the data. The authors state that tumors in PR-SET7 mutant mice arise form ductal /progenitor cells. However, one can envision the scenario that the cre is not 100% efficient so, the tumoral cells could be derived from hepatocytes where PR-SET7 is not deleted and that these repopulate the remaining liver while undergoing an oncogenic transformation. Could the authors quantify the extent of the Cre-deletion? That last point would benefit/require lineage tracing experiment to fully rule out the cell of origin in these tumors. In the absence of this data, the authors should tone down the cell of origin of the tumor. That still doesn't hamper the conclusion of the paper, where SET7 is essential to maintain liver homeostasis and in its absence liver damage is induced, which can contribute to an oncogenic transformation of the tissue.

Referee 2 main point: A major point of the manuscript is that hepatocellular carcinomas in...
PrSET7 ko mice develop from ductal progenitor cells. The line or argumentation is that (a) PrSet7-deficient hepatocytes arrest in cell cycle when they start proliferation (b) ductal cell markers can be detected in regenerative zones of PrSet7 ko livers (c) gene expression profile between hepatocyte-derived HCC progenitors and PrSet7 ko livers is quite different. Although these points are highly suggestive for the ductal progenitor origin of PrSet7 HCCs: they are not entirely conclusive. Especially the point that deletion escaper cells could contribute to HCC cannot easily be excluded. It is clear that the majority of hepatocytes in P45 mice has lost PrSet7. Thus, severe damage, like a partial hepatectomy cannot be compensated by spurious deletion escapers. However, continued low-level damage over 240 days is a different scenario. In support of the deletion escaper hypothesis is actually the fact that in P240 livers PrSet7 is ubiquitously detectable in livers (Fig. E4B) although the cells express high levels of Albumin (Fig. 6D) whose promoter drives Cre expression and should lead to deletion of PrSet7. In other words, PrSet7 clearly escapes deletion in the regenerative areas. Otherwise regenerative processes and development of HCC would probably not be possible due to cell cycle arrest.

Ultimately, lineage-tracing experiments are probably the only way to clearly demonstrate the origin of HCC in PrSet7 ko mice. In absence of a clear demonstration I would recommend to tone down the title of the manuscript and to modulate the discussion accordingly.

Referee 3 main point: The claim is that hepatic progenitors give rise to hepatocellular carcinoma but this is not directly proven using a positive lineage tracing experiment. As such there is a residual concern that there could be an alternative explanation for the results i.e. that the HCCs are due to hepatocytes that were un-recombined by the albumin cre. This comes to my main issue - the title "Oncogenic transformation of differentiating ductal progenitors gives rise to cancer stem cells in hepatocellular carcinoma". My contention is that for such a strong and important claim to be made either a positive tracing technique should be employed, the claim should be modified or the authors should be able to argue or demonstrate that there is zero chance that the HCCs are not arising from an unrecombined hepatocyte that could then undergo ductular change and thereby express all the biliary/ductular markers mentioned in the text (e.g. Sox9, A6 etc., the CD133 staining here is an unusual pattern to me). (Xenografts are performed and again suggest a ductular / progenitor phenotype as does a gene expression profile from the cancer bearing livers. It is unclear to me whether this PR-SET7 deletion is relevant to human HCC and such human data would strengthen the paper.)

Obviously many HCCs have progenitor phenotypes and a growing view is that this may represent phenotypic change rather than reflecting origin. Recent work has suggested using lineage tracing that hepatocytes undergo a ductular change during injury and this view should be taken into account and discussed.

Overall this is a clear paper that relies heavily on immunostaining and negative tracing of progenitors into HCC. How reliable this is depends upon the recombination efficiency.

Response: We agree with the reviewers that proving the ductal origin would be more conclusive if positive lineage tracing experimental data were available. Several models for labeling ductal progenitors (Sox9-Cre-ERT; Ck19-Cre-ERT; HNF1b-Cre-ERT combined with lox-stop-lox reporter mice in the Rosa locus) are available, which have been used in other studies. Unfortunately a Cre-based lineage tracing approach cannot be used for the analysis of our PR-SET7-KO model, since it is based on Cre-mediated recombination of floxed PR-SET7 alleles in postnatal hepatocytes, which would activate the reporter in both hepatic and ductal cells. We note here that the standard PR-SET7 KO mouse is not viable.

We have also thought to use a “negative lineage tracing” approach whereby we label hepatocytes by the fluorescent reporter using the Alb-Cre mice and monitor the complete loss of parental hepatocytes. However, data from such models would not be more conclusive than our original data, since even if 100% loss of labeled cells was observed, one can never exclude the existence and expansion of some parental cells that escape Cre-mediated recombination.

Because of the above restrictions, we have followed the reviewers’ advice and toned down the conclusion concerning the ductal origin of HCC in our model. We have changed the title, the abstract and removed strong statements throughout the text. We have restricted the exposition of our view to the part of the Discussion section where we provide the list of evidence for the possibility of ductal origin. We have kept this as speculation in the Discussion, because we believe that our data are more compatible with the ductal cell expansion-differentiation-oncogenic
transformation mechanism and less with the expansion of hepatocytes that have escaped Cre recombination. This notion is based on the following:

As Referee #2 commented, the “escape” scenario is supported by the appearance of PR-SET7 in P240 hepatocytes, which express Albumin. Thus one would expect that the Alb-Cre transgene will be active and should delete PR-SET7 exon7. However, the expression of transgene-driven Cre is not necessarily activated in all Albumin expressing cells. For example Alb-Cre mice do not express Cre in embryonic hepatocytes where endogenous Albumin expression is high (see also our answer below to Referee #1 point 1). We have measured Cre RNA in P240 PR-SET7 KO livers. They were just above the detection limit as opposed to very high levels at P45 or 120 stage. This can be explained either by assuming that the P240 livers are populated by ductal-like cells, where Alb-Cre is inactive (Figure E1E), even though the cells have differentiated to albumin producing hepatocytelike cells or by assuming that they are hepatocytes (“escapers”), which for some reason do not produce Cre. Both of the above arguments can explain the finding of PR-SET7 detection in P240 KO livers.

We also agree with the comment that partial hepatectomy (PH) is a severe damage, which may not be fully compensated for by spurious “escapers”. We use the data of PH as an argument supporting the “ductal origin” hypothesis, because these mice do live for at least 1 or 2 months after PH and we believe we should have been able to detect at least a few “escaper” hepatocytes in the hundreds of tissue section fields that were examined.

The time course of appearance of A6 or Sox9 single positive and A6/HNF4 or Sox9/HNF4 double positive cells is also difficult to reconcile with the “escaper” hypothesis. Although the possibility that “escaper” hepatocytes first de-differentiate to A6 and Sox9 single positive cells and then differentiate back to hepatocytes cannot be formally excluded, we think that it is quite unlikely to occur in such a synchronous manner.

For the question of Referee #1 (Could the authors quantify the extent of the Cre-deletion?) and Referee #3 (How reliable this is depends upon the recombination efficiency.) we provide the genomic PCR data in the new Figure E1A, showing that deletion of PR-SET7-exon7 in hepatocytes is complete already at P20. Of course the lack of detection of the wt allele (even after 40 cycles of amplification) does not entirely exclude the possibility that a few cells may have escaped Cre recombination.

Additional indirect evidence was provided by comparisons of PR-SET7 inactivation-driven tumors and hepatocyte-derived DEN treatment-induced tumors. We think that this comparison is meaningful, because both tumors are triggered by initial hepatocyte death, while the subsequent steps, including inflammation, fibrosis, Il6-mediated activation of Stat3, are the same in the two models. Sox9-positive and A6-positive cells are represented at much smaller numbers in the hepatocyte derived, DEN-induced tumors than in PR-SET7 KO tumors (new Figure E7A). In addition we detected significant differences in the global gene expression patterns between the two models (new Figure E7B).

Taken together, we think that the above arguments provide strong but not indisputable evidence for the “ductal progenitor origin” hypothesis. As stated above, we agree that in the absence of positive lineage tracing data the possibility of the “escaper” hypothesis cannot be entirely excluded. Therefore we have modulated the text to avoid strong statements regarding the cellular origin issue, but left a paragraph in the Discussion mentioning both alternative views.

Main Issue 2: Further characterization of the cancer stem cell properties

Referee #4 Point 3. The authors need to prove the presence of cancer stem cells in their model. The hierarchical model of cancer stem cells implies that some of cells in the tumor are true cancer stem cells with unlimited proliferation potential, but that they also give rise to a distinct set of cells that are non-tumorigenic. Hence, the authors need to (I) prove the presence of these two cell populations, and (II) show that upon transplantation, purified tumorigenic “cancer stem cells” give rise to more tumorigenic cells as well as phenotypically distinct nontumorigenic cells. This clarification is not only important for proper nomenclature but to determine whether this is truly hierarchically organized tumor (as in HCC) or simply an accumulation of progenitors/stem cells because the extremely high selective pressure that will more or less kill any differentiated hepatic cell.

Response: We provide new data in the new Figure 7E, where we have further analyzed the tumor
xenografts for stem cell markers in xenografts that were isolated 30 days after injection. In these samples we found that A6-positive and CD133-positive cells are greatly reduced in the majority of the sections, while HNF4-positive cells remained constant. Interestingly we could capture several duct-like structures containing A6 single-positive cells. Since A6 single-positive cells are indistinguishable from cholangiocytes, the above pattern demonstrates that phenotypically distinct more differentiated cell types exist in these samples. These findings indicate that the xenografts obtained from our PR-SET7 KO model resemble hierarchically organized tumors.

Point by point responses to the Referees’ comments

Referee #1

Point 1. PR-SET7 deletion in embryonic and adult liver: The authors use a previously generated floxed allele PR-SET7loxP and crossed with several Cre lines. When PR-SET7 is deleted in the embryonic liver (by AlfpCre) the authors demonstrate that this enzyme is critical for normal liver organogenesis. This section is clear and supported by the data. Then the authors evaluate the effect of deleting PR SET7 in later stages. For that an AlbCre line is used. Albeit it is clear that this animals develop a normal liver and do not have the same phenotype of the AlfpCre, the authors state that this is a "adult" model. However, it is well known that this cre line is expressed in liver progenitors. The authors use the word "postnatal" but then consider this one adult liver. Actually the authors themselves show that Alb is expressed at E18.5 (Fig 1C). The authors use the word "postnatal" but then consider this one adult liver. This section needs rewriting to avoid confusion in that line.

Response: We have corrected the text as suggested and use the term postnatal to avoid confusion. The Alb-Cre mouse strain available in our lab (constructed at NIH) expresses the transgene only after birth. We have analyzed Cre expression and recombination efficiency extensively in different previous projects, which showed that Cre expression could be first detected at P1-P4 and gradually increases up to P30. The recombination efficiency showed variations between individual mice and depended on the targeted locus. In some cases complete loss of the targeted allele occurred at P15-P18 (eg. TAF10 locus; Tatarakis et al., 2008), while in others at P35-40 (HNF4 locus; Martines-Jimenez et al., 2010). This demonstrates that transgene expression driven by a composite promoter does not strictly follow the expression of the endogenous gene, most likely because of the effects of potential regulatory elements in the vicinity of the locus where the transgene was inserted. In our case, although albumin expression is high in embryonic hepatocytes, the activation of the promoter in the transgenic mice is activated later.

Another important point concerns the specificity of recombination. Our previous extensive experience with Alfp-Cre and Alb-Cre mice in different contexts shows that Alfp-Cre (which is highly active at early embryonic stages) can act at the hepatoblast stage at E12.5 or earlier. In such cases (if the animal survives) recombination can be detected in both hepatocytes and cholangiocytes. In our experience Alb-Cre is highly hepatocyte specific. This is confirmed by the present study, showing that PR-SET7 was not deleted in the ductal epithelial cells (Figure E1E).

Point 2. Loss of PR_SET7 induces DNA damage and necrosis in dividing cells.

The authors observe that there are 3 areas where liver cells are different: "healthy" (area A), enlarged/damaged (area B), regenerated (Area C). This part of the data is not clear and lacks in depth analysis. According to the authors, the big cells are hepatocytes that are necrotic. They base this statement in 2 pieces of data: the absence of tunel positive cells (which according to them in Figure 2D correspond to infiltrating non-hepatic cell types) and the electronmicroscopy analysis presented in figure 3. However, There is no co-labeling that proves that the "enlarged" cells are or derived from hepatocytes. Markers of co-labeling are missing. Might be useful to the authors to stain the paraffin sections with Alb or cyp or hnf4a, which would facilitate the visualization of the different zones and different sizes of cells. It is difficult to assess which area is which in the immunofluorescence presented.

On the same line, the authors assume that tunel positive cells are infiltrating cells. Again, colabeling is missing there to identify the tunel positive population.

What it can be conclude from the data presented it that the DNA damaged cells stained by gH2AX are alb+ cells, but that these cells are the "big" cells on area B, and that these were derived from damaged hepatocytes, is not clear from the data presented.
Response: We agree that co-labeling would be more informative. Due to the different fixation methods in Tunel and immuno-labeling protocols, co-staining is not possible. However, the large cells can be easily distinguished from other cells in both paraffin and cryosections. Staining with HNF4, gH2AX and Albumin (Figure 2C, 2E) demonstrated that they correspond to damaged hepatocytes. Similarly, the very small cells where negative for the hepatic marker HNF4 and positive for the leukocyte and macrophage markers CD45 and F4/80 (Figure E3A and E3B).

On another line, The big cells have lost the monomethyl mark in K20. Then the authors conclude that this is because they become tri-methylated. But, in figure 3B, every cell from area A, B or C are tri-methylated, can the authors explain that?
Also, Do this areas A, b, C correspond to hepatocytes in zone 1, 2 or 3? (i.e. from Central vein-to portal region)

Response: In the revised version we simplified the description and indicate the areas as Area A, B and C. We avoided zone numbering to avoid confusion. The loss of H4K20 monomethylation from the large necrotic cells and the detection of H4K20 trimethylation is explained by the possible up-methylation of the monomethyl mark (page 7 bottom area). This is not proven experimentally, but we consider as the most logical explanation for our observation.

Point 3. Loss of PR-SET7 induces repopulation of the liver from a ductal progenitor that undergoes oncogenic transformation.
The authors observe a ductal/progenitor response following PR-SET7 deletion. That is an interesting observation clearly supported by the data. Then the authors mention that these ductal/progenitors undergo an oncogenic transformation. This assumption is based on p-STAT3 staining. But, is this activation of the STAT3 pathway occurring in the expanding ductal progenitors? Colabeling is missing.

Response: The Stat3 antibody employed in this study did not work in immunofluorescence staining. We tested several commercial antibodies without success. However, the histochemical staining with DAB substrate (Figure 5B) demonstrates that most parenchymal cells are positive for phospho-Stat3. Whether these are hepatocytes that arise from the differentiation of ductal progenitors or hepatocytes that escaped Cre recombination is discussed in the “Main issues” section. We have modified the text, so that the origin of the parenchymal cells in P240 livers is left open.
Point 4. Is listed in the Main Issue 1 section. We toned down the statements concerning the cell of origin of the tumor.

Referee #2

Main point. Is listed in the Main Issue 1 section. We changed the title and modulated the manuscript as recommended.

Additional point 1: The authors make a point that deletion of PrSet7 is already complete in P20 mice. I could not find data supporting this argument.

Response: We included genomic PCR analysis data (new Figure E1A), which answers the question.

Additional point 2: The analysis of cell duplications in postnatal livers (Figure E1D) is not completely clear to me and needs to explained better. Total DNA in 0.1mg liver should be similar at P20 and P240 if cell density does not change. What is known about the proliferation of individual hepatocytes? Do all hepatocytes divide at some point during their life cycle?

Response: The size of the liver significantly increases between P20 and P240. This comes from both increased cell number and increased cell volume. Because the latter cannot be disregarded, we measured total DNA content in the whole liver. This was achieved by normalization of the DNA content values in 0.1 mg liver with the total weight of the liver. We think that this gives a relatively good estimate on cell numbers, since in the stages after P20 the percentage of hepatocytes in the
total cell population of the liver (including sinusoid cells, immune cells, cholangiocytes etc) is not significantly changed (70-80% by different studies). Our median values suppose that low-rate hepatocyte duplication in postnatal livers occurs homogenously. The mathematical term “median number” of duplications, does not specify whether value 1 represents one duplication of 100% of cells or 2 duplications of half of the cells. We have no reason however to believe that in normal liver hepatocyte populations different duplication potential exist, since in regeneration models like partial hepatectomy all hepatocytes enter the cell cycle.

Additional point 3: What is the penetrance of liver cancer in P240 mice?

Response: The penetrance of liver cancer was 100% as judged by the examination of more than 40 mice at ages 240-300 days. We included this information in page 10 of the revised version.

Additional point 4: In arrested PrSet7 ko hepatocytes H4K20me1 is reduced and H4K20me3 is still present (Figure E2C). The authors argue that loss of H4K20me1 may be due to conversion to me3. How can this result be explained in the light of previous data that demonstrated loss of H4K20me3 upon PrSet7 deletion (e.g. Oda 2009, PMID:19223465; Driskell 2012, PMID:22117221)?

Response: Previous studies have established that PR-SET7-mediated H4K20 monomethylation takes place during G2/M phase, which persists through early G1 phase and drops in S-phase when PR-SET7 protein is degraded (reviewed in Beck et al., 2012). The decrease in S-phase is explained by the conversion of H4K20Me1 to di- or trimethylated states, since the levels of these later modifications do not vary during the cell cycle. A possible explanation for the difference with the abovementioned studies would be the consideration of the state of chromatin before PR-SET7 inactivation, since PR-Set7 is the sole enzyme that can catalyze H4K20 monomethylation. If H4K20Me1 modification did not exist in the majority of histones before PR-SET7 inactivation, since PR-Set7 is the sole enzyme that can catalyze H4K20 monomethylation. If H4K20Me1 modification did not exist in the majority of histones before PR-SET7 inactivation, as is probably the case in most 2.5 dpc embryonic cells), Suv4-20h1/h2 enzymes cannot up-methylate them and H4K20Me3 levels will decrease. If however H4K20Me1 modification existed before PRSet7 inactivation, as is the case in hepatocytes or in other cell types in G1 phase, it can be converted to the trimethyl state.

Referee #3

Main point. Referee #3 had one main point, which is listed in the Main Issue 1 section. We changed the title and modified our claim on the origin of HCC.

Referee #4

Point 1. The presented finding are interesting but there are concerns about relevance and interpretation of the data: (I) Is this a valid model for human HCC? The main mechanism of carcinogenesis is through an escape route after blocking the ability of normal hepatocyte to replicate. However, in human hepatocarcinogenesis differentiated hepatocytes typically exist side by side with progenitor-like cells, and it appears unlikely that such a scenario takes place. In fact, there is typically chronic hepatocyte regeneration/proliferation alongside expansion of proliferating progenitor cells - never a total block of hepatocyte proliferation. (II). Is there a known role for PR-SET7 in human hepatocarcinogenesis? The cited study by Takawa et al. shows increased expression of SETD8 whereas the authors delete PR-SET7. This is not further discussed and seems to be the opposite.

We agree with the reviewer in this point. The mechanism of HCC development in PR-SET7 KO model recapitulates many of the main features of human HCC developing on grounds of hepatocyte damage, but has important unique characteristics that are not seen in human cancer. The main consecutive steps in the pathogenesis of human HCC that are mimicked to different extents by various mouse models are: hepatocyte death – inflammation – fibrosis – ROS accumulation – elevated IL-6 production – STAT3 activation – compensatory proliferation. All the above are observed in the PR-SET7 KO model. The unique characteristic of the PR-SET7 KO model is that in the last step, compensatory proliferation of either hepatocytes (that may have escaped Cre-mediated recombination) or differentiating ductal progenitors results in an HCC composed entirely of cells with Cancer Stem
Cell properties. This is a very unique feature, which makes the model quite useful for studying Cancer Stem Cells. We have discussed this in the original and the revised version. With respect to the expression of PR-SET7 in human HCC, we cited the Takawa paper, which shows that PR-SET7 expression is increased in human HCC biopsies. The increased expression as described by Takawa et al., initially may support proliferation through PCNA methylation. However sustained expression of PR-SET7 during the S-phase would lead to replication stress and arrest in the S-phase (for review see Beck et al., 2012b). Previous results in the literature established that balanced and regulated expression PR-SET7 during the cell cycle is pivotal for genome integrity. Our results in animals are in agreement with this. We mentioned the Takawa paper in this context, because up or down-regulation would eventually lead to cell death and because hepatocyte death is a known triggering event in HCC development.

Point 2. The authors need to include actual data how this model differs from mouse models of HCC in which deletion of key survival genes such as Tak1 or Nemo is ablated. Is a similar escape observed in these models and degree of CSC-like cells in these models or is the PR-SET7 model unique? The generation of a condition that does not allow hepatocytes to replicate is naturally going to promote expansion of populations that escape from this. There is simply no human condition that leads to such a drastic impairment of hepatocyte replication and such a profound selection mechanism. Of note, progenitors in human liver are likely to generate functional hepatocytes whereas progenitor cells in the PR-SET7 model will never give rise to mature cells as their offspring will die immediately. While the presented experimental findings are undoubtedly correct, the question whether the model has relevance for the human situation and can give clues to the origin of human HCC remains unanswered. It is hard to judge whether this is simply an exaggeration of the human situation, or whether it is simply an unphysiological model. Likewise, it should be considered that hepatocytes have the ability to de-differentiate into immature progenitor like cells via Hippo/Yap (Cell 2014).

As we noted above (Point 1 response), we can state only that the HCC developing in PR-SET7 KO mice resembles human HCC (and other mouse models, like TAK1, Nemo or Cyld KO) in the major steps of the pathogenesis of HCC following cell death, but differs in the final cellular composition. It is difficult and premature to judge whether the Cancer Stem Cells in PR-SET7 KO mice are similar to CSCs in human hepatocellular carcinomas. The latter are not well characterized and only few (unfortunately low quality) expression data are available from human CSCs that have been isolated via sorting CD133-positive cell populations. In light of the above, we believe that our thorough expression profiling of CSCs in PR-SET7 KO mice and the finding of interesting gene signatures represents important new information for the hepatic CSC field.

We have not performed comparative analyses with TAK1 or Nemo KO mice. The activation of oval cells has been recently shown to take place in the TAK1 KO model that is dependent on FGF7 signaling (Takashe et al., 2013). Although the potential CSC nature of these cells was not explored in the paper, we speculate that in models where the rate of cell death and compensatory proliferation is high (as in Tak1 KO livers) the contribution of oval cell activation to HCC may be substantial.

In the revised version we included comparisons of gene expression profiles between PR-SET7 KO liver tumors and DEN-induced liver tumors, a widely used HCC model initiated by hepatocyte damage (Revised Figure E7).

In the revised version we also mention the recent paper on the inactivation of the Hippo signaling pathway (Yimlamai et al., 2014).

Point 3. Is listed in the Main Issue 2 section.

Point 4. In view of the above questions, there needs to be a much more thorough characterization of tumors. Many of the PCR markers are progenitor rather than HCC markers. Are these true HCCs? The degree of immature cells suggest that these cells may be different from most HCCs. Comparison of gene expression profiles to human HCC, cholangiocarcinoma (the PR-SET7 could also be a form of intrahepatic cholangiocarcinoma given its proposed cellular origin) and mixed HCC-CC should be added. In addition, more careful characterization of tumors should be performed including collagen IV staining, cytokeratin 19 staining and genomic analysis such as CGH (to understand whether this is a pure expansion of immature cells - or true cancer driven by specific genomic events). The authors should also determine whether there is metastasis to the lung.
as commonly observed in late stage HCC.

We have performed global analysis of gene expression patterns using RNA-sequencing and thorough comparisons with other cell types or HCC models. Comparisons with human HCCs were not very informative, due to the very high heterogeneity between different available human data sets, the different platforms, or controls or normalization approaches used and often the low quality of the deposited data. The high degree of differences in the expression profiles between individual patient samples allows only the determination of altered expression of some common signature genes. Many of them can also be detected in PR-SET7 KO mice. For example, in the very comprehensive study containing high quality data, the authors identified 43 differentially expressed genes in human HCC cohorts, which associate with HCC prognosis (Nault et al, Gastroenterology 2013 vol 145 pp 176). 22 of these were also expressed differentially in PR-SET7 KO tumors. Despite the different platforms used, this is considered a very significant overlap, which points to the existence of common features.

We did not include the comparisons in the paper, because our model is composed mainly of Cancer Stem Cells and therefore the most relevant to our story would be comparison with expression data sets from isolated HCC cancer stem cells. Such data are not available (except one set of very low quality) and we feel that comparisons with total tumor-derived expression profiles would not be a good substitute.

We performed the suggested CK19 staining (Figure E5E). Surprisingly, we did not detect substantial expansion of CK19-positive cells in P240 PR-SET7 KO livers, suggesting that intrahepatic cholangiocarcinoma is not developing in these mice.

We have performed the CGH analysis (Figure E4). The large number of deletions/amplifications detected point to the malignant character of the tumors.

During the course of this study we have examined about 30 mice aged between P240-P300 and not observed macroscopic tumors in the lungs. We have also examined H&E sections from the lungs of 4 mice. We could not detect lung tumors.

Point 5. What is the penetrance in this model? The majority of patients with chronic liver disease and cirrhosis do not develop HCC. If 100% of mice develop HCC, then the model does not reflect the pathogenesis of human HCC. A more valid model might be ablation of PR-SET7 in a subset of hepatocytes, e.g. through a less efficient Cre approach (e.g. Alb-CreERT). Have the authors tested if this be sufficient to trigger cancer? Such a model would also be more physiological than in utero deletion (occurring in most Alb-Cre hepatocytes).

As described above the penetrance of liver cancer was 100%. In our model, the pathogenesis is slow as cell death occurs gradually, when hepatocytes enter the cell cycle to compensate for the loss of the neighbors. We have not tested partial deletion models. In our hands Alb-CreERT mice gives high recombination rates. Attempts to modulate it by lowering tamoxifen dose resulted in high levels of individual variations that compromised reproducibility.

Point 6. The discussion is too lengthy.

We made efforts to shorten it, but after the inclusion of the new data, the length in the revised paper remained similar to the first version.

Minor point 1. It is not clear why the authors use immunosuppressed mice for their transplant models. Hepa1-6 cells can be easily engrafted into syngenic mice. The same should be true for PRSET7 cells. The authors should try this approach.

We agree with the reviewer that the grafting can be done in syngenic mice and there is no need to use immunodeficient ones. Based on pilot injections, we do not expect that tumor growth or tumor composition would differ between syngenic or immunodeficient mice.

Minor point 2. The authors state "postnatal deletion" or "hepatocyte-specific deletion" using the albumin-Cre mice. Deletion occurs in utero and also includes the biliary compartment (possibly progenitors). Hence, the terminology should be altered.

As explained above (Referee 1 point 1 response) only Alfp-Cre mice can drive deletion during the
embryonic stage. Because inactivation often occurs in hepatoblasts, deletion of the targeted exon can be observed in both hepatocytes and cholangiocytes. The Albumin-Cre (Alb-Cre) mice in our models always drive deletion of the targeted alleles at postnatal stages.

Thank you for submitting your manuscript "Spontaneous development of hepatocellular carcinoma with cancer stem cell properties in PR-SET7-deficient livers" for consideration by The EMBO Journal. We have now received the comments of the four referees, which you will find copied below. Three of the four referees now suggest publication of your manuscript. Please note that referee #3 did not submit a written explanation, but let us know that the revised version is now suitable for publication in the EMBO Journal.

We have to note, however, that referee #2 specified that the origin of HCCs in the PR-SET7 knockout has not been conclusively determined. Similarly, referee #4 maintains that a number of the originally raised concerns will have to be more clearly addressed before publication in the EMBO Journal can be granted. These residual concerns were already raised in the initial review, and the editorial team strongly thinks that addressing them carefully is important for the overall interpretations that can be drawn from this study. We came to the conclusion that a second final touch-up is necessary that attends to the open points with appropriate careful textual changes for final acceptance of the manuscript.

To be clearer about the requirements for a final acceptance for publication:
In the face of referee #4's concerns, we advice you to introduce a short passage in the main text on the Cre lines used (where and when they delete in hepatocytes and cholangiocytes, similar to your detailed response to the referees). We also advice you to reference alternative models more clearly and outspoken (points 1/3), and to add a statement on the state of the hepatocytes generated in the model (point 4) as well as to more critically interpret the nature of the tumor cells (point 5). The only point that needs some experimental attention is point 2: we foresee that a test of PR-SET7 deletion in the biliary duct using the two driver lines should be straight forward, take advantage of existing samples and be added to Fig E1.

Secondly, although you already mentioned this in the response to the referees (which will also be published online), we would appreciate if you introduced a small passage also in the main manuscript acknowledging the formal "escaper" hypothesis. We understand that you discuss this issue, but at the moment you decisively maintain that this "escaper" possibility "can be excluded" supported by some reasonable considerations (p.18), in agreement with the referees' overall concerns during round 1 of the revision and with referee #4 maintaining the concern, we favor a more balanced/cautious statement here.

There are some other formalities: We would like to ask you to provide a detailed methodological description of E1A in the manuscript (isolation; cycle number and sensitivity). Please also move all materials and methods (including references) from the expanded data files to the main text methods section and indicate accession numbers for all the expression analyses including the RNAseq data (where missing).

Finally, I will separately be sending you an Excel sheet which we would like to ask you to fill in completely and upload together with your revised manuscript as "supplementary file": This Excel sheet comprises a "checklist" which serves to report more transparently on statistics and methods used in publications, as well as on details of animal/human models (where applicable) or on and data accessibility. The checklist is in line with the recommendations made by the NIH hosted working group on publishing data (which met in June 2014 and which The EMBO Journal signed alongside around 30 other leading journals). Many thanks for providing this additional set of information.

Please do contact me if you have any questions regarding this decision.
REFEREE REPORTS

Referee #1:

In my original review I had appreciated the elegance of the work described albeit I had raised some concerns mainly regarding the claim that HCC PER-Set7 tumors were derived from duct/progenitor cells. In addition, I had questioned whether the necrotic cells where the enlarged hepatocytes. In the revised version the authors address both points. In my view, my criticisms have been addressed and the manuscript has been improved.
I would recommend the manuscript from Nikolaou et al., to be published by EMBOJ.

Referee #2:

The major issue of the initial submission, the origin of HCC in Prset7 ko mice, could not be solved experimentally. Therefore the authors decided to follow the reviewers' advice to tone down the conclusions and to change the title of the manuscript. The additional minor points were addressed satisfactorily. Thus, I would recommend publication of this manuscript.

Referee #4:

The authors have responded to many of the previous criticism, and toned down many of their statements. However, there are still a number of issues that have not been clearly addressed and will be a source for confusion. Currently, the manuscript will not really advance the field as the model is still not sufficiently evaluated, and questions not clearly answered.

1. The abstract should not state "hepatocyte-specific" deletion and "Inactivation at postnatal stages" as the Albumin-Cre mice delete in the biliary compartment and as deletion occurs prenatally (to a lesser degree than in Alfp-Cre mice, but it does occur). There is ample evidence for both in a large body of literature. These statements and the nomenclature should be corrected throughout the manuscript. This point had already been mentioned in the previous review.

2. In regards to above criticism, please determine whether PR-SET7 is deleted in the biliary tract and include this data in the manuscript.

3. The introduction is misleading as it cites studies such as Sox9 tracing (Furuyama et al.) that are not accepted by the great majority of the field (other tracing methods clearly contest these data), and - more importantly - as a number of recent publications show that hepatocytes (e.g. from the Stanger and Willenbring groups) are the sole source of newly generated hepatocytes in mice. A clear statement that hepatocytes appear to be the dominant source of hepatocytes and that LPC play a limited or non role in this process in mice - is required with appropriate references.

4. The author need to address the issue of escape from Cre-mediated recombination more thoroughly. Typically, the biliary/progenitor compartment is deleted by Alb-Cre. Hence, detection of intact PR-SET7 and H4K20Me staining argues for escape. Even if the PI believes that Alb-Cre does not delete in this compartment (which is unlikely), the fact that HNF4 and Albumin are detected in the supposed offspring of cells implies that Alb-Cre should become active and delete PR-SET7 - which it doesn't. In either scenario, there appears to be escape from Alb-Cre-mediated deletion that should happen in most hepatocytes. If the authors still want to argue that escape does not take place, they do need to conclude that progenitors do not generate completely normal hepatocytes in their model. Either it is escape or the progeny are not real hepatocytes - there is no other possibility.

5. The characterization of the "cancer stem cells" is insufficient. Demonstration that that they can differentiate into non-tumorigenic cells is essential (e.g. by transplanting differentiated off-spring devoid of CSC into mice and showing that they essentially are not able to establish tumors; alternative strategies are also acceptable if they provide similar evidence). If the authors cannot do
this and if all cells in this model are "stem cells", then this is not a tumor with hierarchical organization as suggested in the cancer stem cell model, and the term "cancer stem cell" should be avoided; it could just be that this is a tumor almost exclusively composed of immature cells, but not true cancer stem cells. Fig. 7E in this regard is not sufficient as there is not only not quantification but a lack of functional experiments showing the tumor-forming capacity of these cells.

Response to the Reviewers

In the new version we followed the Editorial advice and made the following textual changes:

1. To address point 3 and part of point 4, we refer to the new papers from the Ben Stanger and Holger Willenbring labs (Schaube et al, 2014; Yanger et al, 2014) in the introduction and discussion sections (page 4 and 19).

In the Discussion (page 19 and 20) we included new text clearly stating that the alternative 'escaper hepatocyte' hypothesis is not formally excluded by the present data: page 19... “On the other hand, since direct lineage tracing experiments cannot be performed in the Cre-lox system mediated PR-SET7 KO model, the possibility that some hepatocytes escape Cre-mediated recombination and due to the strong selective pressure, de-differentiate and repopulate the liver cannot be entirely excluded. This possibility is also supported by recent studies demonstrating that existing hepatocytes and not progenitors generate new hepatocytes in different injury-mediated liver regeneration models (Schaub et al, 2014; Yanger et al, 2014).”

Page 20... “We note however, that although the above arguments indirectly support the progenitor origin of hepatocytes in P240 PR-SET7ΔHepA livers, they do not provide unequivocal evidence to rule out ‘escaper’ hepatocyte origin.”

2. To address point 1 and 2 we highlight the data in Figure E1E, clearly showing that in PR-SET7lox/lox-Alb-Cre mice PR-SET7 expression is lost from all hepatocytes and not from biliary epithelial cells (marked by the arrow). This answers the issue of hepatocyte specificity raised in points 1, 2 and 4.

We have also included the original reference of the source of Alb-Cre mice (Yakar et al. 1999) that was missing in the original manuscript. In this paper it was determined that deletion of the floxed Igf1 allele was observed from postnatal day 10. Of course, the timing of deletion is probably affected by the genomic locus (as suggested by our extensive previous experience) and the mouse strains. We included a sentence in the Methods section stating that: “In the mixed C57Bl6/CBA background, Alb-Cre mice expressed detectable amounts of the transgene only after birth.”

With respect to the timing of recombination, we think that it is clear that in the present case potential prenatal deletion mentioned by the reviewer does not occur or is negligible (we can provide genomic DNA PCR data, if necessary). If prenatal deletion occurred we expect to have had a lethal phenotype like that observed with Alfp-Cre mice as described in Figure 1.

3. With respect to the nature or the state of the hepatocytes (Point 5) and the tumors in P240 PR-SET7ΔHepA livers, we provide a quite thorough phenotypic and molecular characterization in this paper (staining with hepatic markers and global gene expression pattern). The analysis demonstrates that they are abnormal cancerous hepatocytes “with unique characteristics combining hepatoblast and ductal progenitor cell-like features” (last paragraph of Discussion).

We do not state that the tumors in PR-SET7 KO mice display a hierarchical organization. Instead, we emphasize that our data suggest that these tumors are mainly composed of cancer stem cells. Our graft experiments demonstrate that they can give rise to tumors, containing stem cell marker-expressing cells and more differentiated cells.
While this is mentioned already in several parts of the paper, we revised the text in page 20 of the Discussion to be more specific:

“...P240 $PR\text{-SET7}^{\Delta}$ hepatocytes express several markers such as Sox9, CD133, EpCAM, Cd44, which are used to identify CSCs, and oncofetal marker genes, which are used to identify HCC. These features suggest that P240 $PR\text{-SET7}^{\Delta}$ hepatocytes resemble transformed immature cancer cells characteristic of highly aggressive tumors. Their potential to self-renew in culture and give rise to tumors containing stem cell marker-expressing cells and more differentiated cells in xenografts demonstrate that they correspond to cancer stem cells.”

Other text change includes:
Addition a sentence to the legend of Figure E1 about the PCR primers and the number of PCR cycles employed.