Hepatic stellate cells limit hepatocellular carcinoma progression through the orphan receptor endosialin

Carolin Mogler, Courtney König, Matthias Wieland, Anja Runge, Eva Besemfelder, Dorde Komljenovic, Thomas Longerich, Peter Schirmacher, and Hellmut G. Augustin

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Review timeline:

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: Roberto Buccione

1st Editorial Decision   22 November 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three reviewers whom we asked to evaluate your manuscript.

As you will see the three reviewers are quite positive although they do raise some concerns on your manuscript, which I would basically summarise as follows. Many comments are focused on improving presentation of data and providing much better experimental details, both very important aspects in our quest at EMBO Molecular Medicine to improve the reproducibility of published findings. Reviewer 2, instead notes that your claim that IGF2 is a major mediator of endosialin loss in HSCs is not fully supported by the data and would like you to attempt further experimentation to solidify this aspect. S/he also has a concern on the cytokine array experiment and suggests validating some key cytokines.

In conclusion, while publication of the paper cannot be considered at this stage, we would be pleased to consider a revised submission, with the understanding that the Reviewers' concerns must be addressed including further experimentation as mentioned above. Eventual acceptance of the manuscript will entail a second round of review.

Reviewer 1 also suggests the reorganisation of your manuscript as a Research Article rather than a Report. I would not necessarily ask for this but if you feel that full compliance with the numerous requests would justify this transformation, I would have no objection. Needless to say, I would in such case suggest you to upgrade a couple of the most relevant figures from appendix to main.

I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

All results are potentially interesting and novel. However there are a few major issues that need to be addressed before the paper is fully convincing.

Referee #1 (Remarks):

The manuscript by Mogler et al. aimed to investigate the role of hepatic stellate cell-expressed endosialin in cell proliferation during hepatocarcinogenesis. It is a very concisely written paper and presents results in a logical order, highlighting a negative correlation of endosialin expression and HCC tumour cell proliferation. The presented results are convincing, however sometimes the manuscript would have benefited from more background information or additional experiments to round off the story.

Comments:

Introduction: Very brief and a lot more background could be provided with regards to HCC tumour niche development and cellular crosstalk of key regulators to set the scene for endosialin and the rest of the manuscript.

Results:

Figure 1: The authors show immunohistochemical stainings of human liver biopsy material for endosialin and Ki67. No patient information is provided. What is the chronic liver injury background that the HCCs developed on? Viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease? Different aetiologies may induce divergent histological features and contribute to the considerable regional variations that the authors are describing. It is stated that only hepatic stellate cells and portal myofibroblasts express endosialin but it is not possible to derive this from the shown images. Dual immunofluorescence for endosialin and respective cell markers would be more convincing since it cannot be ruled out that other cell types in the portal niche induce endosialin under appropriate conditions (such as liver progenitor cells for example).

Figure 2: Methods details for PCNA staining are missing. Manufacturer details are missing for detection with the biotin-peroxidase complex. Western Blot primary antibody details missing. Q is difficult to interpret due to a lack of labelling and the result shown for background fluorescence in between the two experimental groups.

Figure 3: The authors describe an altered morphology in HSCs when endosialin was silenced, however Panel A is difficult to interpret due to suboptimal quality and no higher magnification insert provided. Also, in the text the altered morphology is referred to in Figure 3A and B, however B shows the proliferation rate, not the morphology as such. There are not enough details provided for the EDU assay and even with the manufacturer's details, it is not possible to find the correct assay and potentially replicate the results. For LX2 culture it is described that the cells were either grown in 2% or 10% FBS, which is a big difference but no details are provided as to which concentrations were chosen for which assay.

Overall, this work has a lot of potential and the results are potentially exiting but the manuscript feels preliminary due to the lack of provided details and lack of discussion.

I do believe that this report would be better suited as a full research article to be able to provide more background as well as experimental details and make the results more convincing.

Referee #2 (Comments on Novelty/Model System):

the manuscript uses a mouse model recently reported by their lab, cell lines and studies of human tissues. This is very suitable and the claims are supported by the data.

Referee #2 (Remarks):

Endosialin is an orphan receptor that is expressed in the liver in disease states, mostly in hepatic stellate cells (HSCs). These cells are known to play important roles fibrosis and in regulating liver regeneration. However, their role in HCC is not clear and they were shown to play both positive and negative roles. The mechanisms that impart such negative or positive roles on HSCs are not known.

Mogler et al have previously shown that endosialin is an important regulator of HSCs in liver fibrosis and regeneration. They now go on to reveal that endosialin is a key molecule that imparts an anti-oncogenic role on HSCs. The article reveals that loss of endosialin changes the phenotype of HSCs, and they assume a phenotype characterized by secretion of several pro-proliferative cytokines that are known to be important for HCC progression. The cell line and well conducted mouse studies are accompanied by a convincing set of human data that goes along with their claim.

Comments:

In my mind the phenotypic switch (i.e. that HSCs can assume "good" vs. "bad" phenotype) should be made more prominent in the abstract, discussion as well as the graphic abstract in fig 3J.

On the other hand, the authors seem to claim that the data showing that silencing IGFR1 in hepatocytes (Fig 3H) results in reduced HCC proliferation proves that IGF2 is a major mediator of the effect of endosialin loss in HSCs. While the data provides correlation it does not prove this. To do so, they should overexpress IGF2 in endosialin sh cells, and use those in co culture. I am not sure this experiment will work, as there are many other mediators. The bottom line - the conclusions should be softened (i.e. "indicating" changed to "supporting the possibility" or the like).

The cytokine array experiment (fig 3I) is very important as it shows a real phenotype switch in HSCs upon endosialin knockdown. It is not clear how many times this was repeated. If only once, then some of the highly differentially regulated cytokines should be assessed in an additional manner (specific ELISA, real time PCR if known to be regulated at mRNA level etc.).

It is not clear if the data presented in fig 2a-c is statistically significant.

Referee #3 (Remarks):

This is an interesting and well-executed study that reports on the functional contribution of hepatic stellate cells (HSC) derived endosialin expression in hepatocarcinogenesis. Human hepatocellular carcinoma (HCC) tissues were used to establish the correlation between HSC derived endosialin expression and tumor progression. The authors also used genetically engineered endosialin KO mice to test their hypothesis: HSC endosialin promote hepatocyte proliferation, implicating IGF2 paracrine signaling. Finally, a knock down culture system was used to define the communication between HCC and HSC. The study is well constructed and thoroughly tested. Pending minor clarifications, this manuscript thus offers a novel insight on hepatocarcinogenesis.

1. The authors previously reported that HSC derived endosialin is implicated in liver fibrosis and regeneration (Mogler et. al., 2015). This should be better emphasized in the introduction of the manuscript as highly relevant for the hypothesis testing brought forth in this manuscript.
2. Figure 1, panel C, the staining is difficult to appreciate; please consider presenting a higher magnification image. The figure legend lettering appears to be incorrect, please verify.
3. In Extended view Fig. 1, are the authors implying that there are heterogeneous expression pattern/level on TMA? Have they correlated these differences in expression with respect to HSC content in TMA sections?
4. Figure 2C: are these differences statistically different? For the data presented in Figure 2, The number of animals used in each group is not listed in the figure legend.
5. What is the meaning of figure 2P-Q? Is it supportive of the data presented in M-O?
6. Figure 3, please indicate the percent down regulation of endosialin in the knockdown strategy?
7. Figure 3E. Is there a difference in % Edu+ HCC tumor cells between none-treated (Just HCC tumor cells) vs treated with CM ns EN? Please note the poor quality of the image in panel 1 renders it difficult to appreciate the results.

1st Revision - authors' response   27 February 2017

Response to Reviewers' comments

Reviewer #1

COMMENT ON NOVELTY/MODEL SYSTEM: All results are potentially interesting and novel. However, there are a few major issues that need to be addressed before the paper is fully convincing.

RESPONSE TO NOVELTY/MODEL SYSTEM: We sincerely appreciate the reviewer's overall positive assessment of our work and would like to thank him/her for the thoughtful suggestions to further advance the manuscript. All comments of the reviewer have been addressed in additional experiments and editorial revisions. The changes to the manuscript are outlined in detail below.

GENERAL COMMENT: The manuscript by Mogler et al. aimed to investigate the role of hepatic stellate cell-expressed endosialin in cell proliferation during hepatocarcinogenesis. It is a very concisely written paper and presents results in a logical order, highlighting a negative correlation of endosialin expression and HCC tumor cell proliferation. The presented results are convincing, however sometimes the manuscript would have benefited from more background information or additional experiments to round off the story.

RESPONSE TO GENERAL COMMENT: We have redrafted the introduction and discussion sections to include more background information. Following the reviewer's suggestions, we have performed additional experiments that are now included in the main figure and the supplemental material of the revised manuscript and outlined in detail below.

COMMENT 1: Introduction: Very brief and a lot more background could be provided with regards to HCC tumor niche development and cellular crosstalk of key regulators to set the scene for endosialin and the rest of the manuscript.

RESPONSE 1: The '*Brief Report*' format limits the overall length of the manuscript. However, by expanding the introduction and discussion, we have aimed at addressing the reviewer's concerns.

COMMENT 2: Figure 1: The authors show immunohistochemical stainings of human liver biopsy material for endosialin and Ki67. No patient information is provided. What is the chronic liver injury background that the HCCs developed on? Viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease? Different etiologies may induce divergent histological features and contribute to the considerable regional variations that the authors are describing. It is stated that only hepatic stellate cells and portal myofibroblasts express endosialin but it is not possible to derive this from the shown images. Dual immunofluorescence for endosialin and respective cell markers would be more convincing since it cannot be ruled out that other cell types in the portal niche induce endosialin under appropriate conditions (such as liver progenitor cells for example).

RESPONSE 2: Following the reviewer's suggestion, we compared the amount of Endosialin to the corresponding clinical data for the samples used for this study. We do not see a correlation with regard to the etiology of the HCC samples (13 whole slide samples of HCC including 5 cases of viral hepatitis [4x HBV, 1x HCV], alcoholic steatohepatitis/ASH [4 cases] or non-alcoholic steatohepatitis/NASH [4 cases]). To exclude negative data resulting from a small sample number, we increased the number of HCC samples including another cohort of HCC samples (20 samples of fresh frozen HCC samples: 7x viral hepatitis [4x HBV/3x HCV], 7x ASH, 5x NASH, 1x unknown). Again, Endosialin levels were independent of the etiology. We included this in the results section of the revised manuscript (page 5). We interpret these findings (also in view of our previous work on Endosialin expression during liver fibrosis [Mogler et al., EMBO Mol Med, 2015) as indicating that activation of hepatic stellate cells and increase of Endosialin expression seems to be independent of the cause of damage: Any kind of acute or chronic liver injury will lead to an activation of hepatic stellate cells which is accompanied by Endosialin expression. Moreover, chronic liver damage is often multifactorial: The main cause of liver damage might be aggravated by additional risk factors (e.g. patients with history of chronic alcohol abuse might suffer from metabolic syndrome as well or e.g. patients with history of chronic viral hepatitis might experience additional drug induced liver injury [DILI]).

Addressing the second point of the reviewer: We have previously analyzed in substantial detail

Endosialin expression in the normal and fibrotic liver (Mogler et al., EMBO Mol Med, 2015). We have in this study analyzed gene expression in isolated liver cell populations (qPCR, IHC, IF) and could robustly trace Endosialin expression to cells from the mesenchymal lineage with quantitatively highly preferential expression by hepatic stellate cells and portal (myo-)fibroblasts. We have not yet performed Endosialin genetic fate mapping experiments and can therefore not formally exclude that some smaller progenitor cell populations could express Endosialin. Yet, quantitatively speaking, we are confident that hepatic stellate cells and portal (myo-)fibroblasts are the primary Endosialin expressing cells in the liver. To accommodate the reviewer's concern, we have employed some more cautionary wording ('*predominantly*') in the manuscript (page 4).

COMMENT 3: Figure 2: Methods details for PCNA staining are missing. Manufacturer details are missing for detection with the biotin-peroxidase complex. Western Blot primary antibody details are missing. Q is difficult to interpret due to a lack of labelling and the result shown for background fluorescence in between the two experimental groups.

RESPONSE 3: We included the missing information (revised methods section in the expanded view material). Concerning the Western blot analysis shown in Fig. 2Q of the original manuscript, we apologize for the misleading representation of the data in the original manuscript. We used the GE Healthcare Amersham Western Blot machine (panel on the left in Fig. 2Q), which automatically quantifies the amount of detected target protein in relation to total protein. This automatic quantification was shown on the right side of Fig. 2Q. The data are supportive of the immunohistochemical images shown in 2M and 2N. We therefore transferred these data to the supplemental material and changed the arrangement (quantitation below bands [expanded view Fig. 7]).

COMMENT 4: Figure 3: The authors describe an altered morphology in HSCs when endosialin was silenced. However, panel A is difficult to interpret due to suboptimal quality and no higher magnification insert provided. Also, in the text the altered morphology is referred to in Figure 3A and B. However, B shows the proliferation rate, not the morphology as such. There are not enough details provided for the EdU assay and even with the manufacturer's details, it is not possible to find the correct assay and potentially replicate the results. For LX2 culture it is described that the cells were either grown in 2% or 10% FBS, which is a big difference but no details are provided as to which concentrations were chosen for which assay.

RESPONSE 4: We have replaced the images with higher quality images to show Endosialin-silenced and mock-transfected LX2 cells. Following the reviewer's suggestions, we also included a higher magnification image in the main figure (Fig. 3A of revised manuscript). The altered morphology in Endosialin-silenced cells vs. mock-transfected cells includes more abundant cytoplasm and slightly enlarged nuclei (included in revised manuscript). As suggested, additional detail on the EdU assay has been included in the method section (revised expanded view method section). The EdU assays are a reliable surrogate marker to assess the proliferation of cells (HSC and Huh7) in vitro (e.g. as previously shown in Mogler et al., EMBO Mol Med, 2015 or Hu et al., Science, 2014). All experiments have been performed using 2% FCS and performed 3 times independently with 3 biological replicates for each experimental condition (included in methods section). After performing the EdU staining protocol according to the manufacturer´s protocol, ten images per well and condition were randomly taken. Images were then automatically quantified by two different investigators (C.M. & C.K.) using Image J software (FIJI).

COMMENT 5: Overall, this work has a lot of potential and the results are potentially exiting but the manuscript feels preliminary due to the lack of provided details and lack of discussion.

RESPONSE 5: Expanding the manuscript by addressing the reviewer´s very helpful suggestions leading to the inclusion of the results of additional experiments and editorial revision of manuscript, we believe that the manuscript has substantially grown and will now make a strong contribution.

COMMENT 6: I do believe that this report would be better suited as a full research article to be able to provide more background as well as experimental details and make the results more convincing.

RESPONSE 6: We sincerely appreciate the reviewer's suggestion to expand this manuscript towards a full research article. The work is timely, competitive and has the potential to conceptually advance

the field. We would consequently like to communicate the findings rather earlier than later. We therefore opted for the '*Brief Report*' format which we consider quite suitable towards this end. We do believe that expansion of the manuscript during the revision have significantly strengthened the study to now make it a "round story".

Reviewer #2

COMMENT ON NOVELTY/MODEL SYSTEM: The manuscript uses a mouse model recently reported by their lab, cell lines and studies of human tissues. This is very suitable and the claims are supported by the data.

RESPONSE TO NOVELTY/MODEL SYSTEM: We sincerely thank the reviewer for his/her very positive feedback.

GENERAL COMMENT: Endosialin is an orphan receptor that is expressed in the liver in disease states, mostly in hepatic stellate cells (HSCs). These cells are known to play important roles fibrosis and in regulating liver regeneration. However, their role in HCC is not clear and they were shown to play both positive and negative roles. The mechanisms that impart such negative or positive roles on HSCs are not known. Mogler et al have previously shown that endosialin is an important regulator of HSCs in liver fibrosis and regeneration. They now go on to reveal that endosialin is a key molecule that imparts an anti-oncogenic role on HSCs. The article reveals that loss of endosialin changes the phenotype of HSCs, and they assume a phenotype characterized by secretion of several pro-proliferative cytokines that are known to be important for HCC progression. The cell line and well conducted mouse studies are accompanied by a convincing set of human data that goes along with their claim.

RESPONSE TO GENERAL COMMENT: We again would like to thank the reviewer for his/her positive assessment of our work.

COMMENT 1: In my mind the phenotypic switch (i.e. that HSCs can assume "good" vs. "bad" phenotype) should be made more prominent in the abstract, discussion as well as the graphic abstract in fig 3J.

RESPONSE 1: We thank the reviewer for his/her suggestion with which we fully agree. We have highlighted this issue and expanded the introduction and discussion of the revised manuscript accordingly.

COMMENT 2: On the other hand, the authors seem to claim that the data showing that silencing IGFR1 in hepatocytes (Fig 3H) results in reduced HCC proliferation proves that IGF2 is a major mediator of the effect of endosialin loss in HSCs. While the data provides correlation it does not prove this. To do so, they should overexpress IGF2 in endosialin sh cells, and use those in co culture. I am not sure this experiment will work, as there are many other mediators. The bottom line - the conclusions should be softened (i.e. "indicating" changed to "supporting the possibility" or the like).

RESPONSE 2: We fully agree with the reviewer that the correlative finding is not sufficient to make a causal claim. Endosialin-silenced HSC produce more IGF2, which we hypothesize drives HCC proliferation. As such, an IGF2 gain-of-function experiment would not be reasonable. Yet, to accommodate the reviewer's concern, we have redrafted the text accordingly and used more cautionary wording.

COMMENT 3: The cytokine array experiment (Fig 3I) is very important as it shows a real phenotype switch in HSCs upon endosialin knockdown. It is not clear how many times this was repeated. If only once, then some of the highly differentially regulated cytokines should be assessed in an additional manner (specific ELISA, real time PCR if known to be regulated at mRNA level etc.).

RESPONSE 3: We included the cytokine array experiment to highlight the observation that IGF2 may be a primary mediator of the HSC Endosialin effects on hepatocarcinogenesis, but probably not the only one. The array experiment was performed several times with various passages of HSC. Depending on the HSC passage, there is some variation in the array results and the hierarchy of the

most regulated cytokines. We therefore performed additional EdU stimulation experiments with Huh7 tumor cells for the most robustly regulated cytokines identified in the arrays, namely CCl5 and RBP4, for which pro-proliferative effects of both, CCl5 and RBP4 have previously been reported for HCC cells. These experiments essentially identified the same proliferative effect as observed for IGF2 (included in expanded view figure 11). The text has been correspondingly rephrased to explicitly state that IGF2 may be a major, but not the only contributor to the observed effects.

COMMENT 4: It is not clear if the data presented in fig 2a-c is statistically significant.

RESPONSE 4: The data presented in Fig. 2C showed the percentage of mice presenting with CT-detectable tumor nodules. The data (as indicated) are indeed not statistically significant (using Fisher´s test: p=0.084 for 6 weeks and p=0.096 for 7 weeks). This graph was supposed to support the hypothesis that loss of Endosialin plays a role in early tumor development. Yet, we agree with the reviewer that the simple tumor / no tumor analysis is too crude for a meaningful quantitation. We have therefore quantitated the number of CT-detectable nodules >0.5ml, which yields meaningful readouts and demonstrates the highly significant effect of Endosialin absence or presence on tumor growth (Fig. 2C of revised manuscript).

Reviewer #3

GENERAL COMMENT: This is an interesting and well-executed study that reports on the functional contribution of hepatic stellate cells (HSC) derived endosialin expression in hepatocarcinogenesis. Human hepatocellular carcinoma (HCC) tissues were used to establish the correlation between HSC derived endosialin expression and tumor progression. The authors also used genetically engineered endosialin KO mice to test their hypothesis: HSC endosialin promote hepatocyte proliferation, implicating IGF2 paracrine signaling. Finally, a knock down culture system was used to define the communication between HCC and HSC. The study is well constructed and thoroughly tested. Pending minor clarifications, this manuscript thus offers a novel insight on hepatocarcinogenesis.

RESPONSE TO GENERAL COMMENT: We sincerely thank the reviewer for his/her very positive overall feedback.

COMMENT 1: The authors previously reported that HSC derived endosialin is implicated in liver fibrosis and regeneration (Mogler et. al., 2015). This should be better emphasized in the introduction of the manuscript as highly relevant for the hypothesis testing brought forth in this manuscript.

RESPONSE 1: We have expanded the introduction to better highlight the results from our previous work, which have led to the experimental hypothesis of the current manuscript.

COMMENT 2: Figure 1, panel C, the staining is difficult to appreciate; please consider presenting a higher magnification image. The figure legend lettering appears to be incorrect, please verify.

RESPONSE 2: Endosialin expression in dysplastic nodules was weak and mostly restricted to the intralesional hepatic stellate cells. According to the reviewer´s suggestion, we have included a high magnification image (400x) in the main revised figure. As additional information, two lower magnification images (200x) have been included in the supplementary material (revised Fig. 1 and expanded view Fig. 1). Furthermore, we thank the reviewer for alerting us of the wrong labeling in the legend. This has been corrected.

COMMENT 3: In Extended view Fig. 1, are the authors implying that there are heterogeneous expression pattern/level on TMA? Have they correlated these differences in expression with respect to HSC content in TMA sections?

RESPONSE 3: Endosialin expression in slides of whole tumor section shows considerable variability with most pronounced expression in the periphery of tumors (e.g., as shown in Fig. 1G and 1H). The small punches of TMAs are really not suitable to appreciate this variability. We therefore limited the TMA analysis to positive/negative analysis. Moreover, TMA punches are mostly taken from the tumor center and less frequently from the tumor periphery. It may therefore not be surprising that overall Endosialin expression appeared to be less prominent in the TMAs

compared to the whole tumor slides. We have previously extensively studied Endosialin expression in non-neoplastic, fibrotic and cirrhotic liver (Mogler et al, EMBO Mol Med, 2015). Here too, we saw that Endosialin is diffusely expressed by activated hepatic stellate cells.

COMMENT 4: Figure 2C: Are these differences statistically different? For the data presented in Figure 2, the number of animals used in each group is not listed in the figure legend.

RESPONSE 4: This comment was also made by reviewer 2 (comment 4): The data presented in Fig. 2C showed the percentage of mice presenting with CT-detectable tumor nodules. The data (as indicated) are indeed not statistically significant (using Fisher´s test: p=0.084 for 6 weeks and p=0.096 for 7 weeks). This graph was supposed to support the hypothesis that loss of Endosialin plays a role in early tumor development. Yet, we agree with the reviewer that the simple tumor / no tumor analysis is too crude for a meaningful quantitation. We have therefore quantitated the number of CT-detectable nodules >0.5ml, which yields meaningful readouts and demonstrates the highly significant effect of Endosialin absence or presence on tumor growth (Fig. 2C of revised manuscript). Additionally, we have included the number of animals for each group and time point in the figure legend (n=10 knockout and n=12 wildtype animals per group and time point).

COMMENT 5: What is the meaning of figure 2P-Q? Is it supportive of the data presented in M-O?

RESPONSE 5: Figure 2P-Q of the original manuscript showed Western blot analyses of the proliferation marker PCNA in Endosialin knockout and wildtype tumor-bearing livers. It is indeed supportive of the immunohistochemical stainings shown in Fig. 2M-O. The data are normalized to total protein (scanning and evaluation was automatically done using the Amersham Western Blotting System GE Healthcare [details in methods section]) and show the quantitation of PCNA protein of each sample shown on the left. As this graphical arrangement seems to be somewhat confusing (see also comment 3 of reviewer 1), we rearranged the graphical presentation and transferred the Western blot data to the expanded view section
(expanded view Fig. 7).

COMMENT 6: Figure 3, please indicate the percent downregulation of endosialin in the knockdown strategy?

RESPONSE 6: The knockdown of Endosialin was consistently more than 90% (see graph on right from 3 different transfection rounds with 3 biological replicates each; shown in S.E.M.). This information is included in the text of the methods section
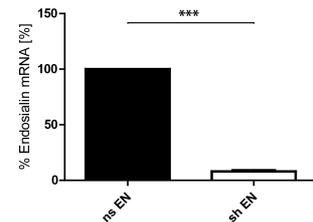
COMMENT 7: Figure 3E. Is there a difference in % EdU+HCC tumor cells between non-treated (just HCC tumor cells) vs. treated with CM ns EN? Please note the poor quality of the image in panel 1 renders it difficult to appreciate the results.

RESPONSE 7: The images in panel 1 have been replaced (see also comment 4 by reviewer 1). As for the effect of conditioned medium from non-Endosialin-silenced HSC on HCC proliferation vs. HCC proliferation without conditioned medium (strictly using the same medium conditions [2%FCS/low glucose DMEM medium]), the reviewer is raising an interesting point: Non-Endosialin-silenced conditioned HSC medium indeed exerts a moderate inhibitory effect on HCC proliferation. This is an effect that is independent of Endosialin and we opted to not include this in the current manuscript, because it is focus of ongoing experiments.

2nd Editorial Decision   13 March 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine.

We have now received the enclosed reports from the reviewer who was asked to re-assess it. As you will see s/he is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final editorial amendments:

1) As per our Author Guidelines, the description of all reported data that includes statistical testing

must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the ACTUAL p VALUE for each test (not merely 'significant' or 'P < 0.05').

2) Please provide 5 keywords in the title page.

Please submit your revised manuscript within two weeks. I look forward to receiving a revised form of your manuscript as soon as possible so that we can proceed with formal acceptance

***** Reviewer's comments *****

Referee #1 (Remarks):

All issues were adequately addressed in the revision.

2nd Revision - authors' response   13 March 2017

Thank you very much for notifying us that our revised manuscript entitled "*Hepatic stellate cells limit hepatocellular carcinoma progression through the orphan receptor endosialin*" is acceptable for publication in *EMBO Molecular Medicine* pending minor additional revisions (additional information on details of statistical analysis [exact p values, names of employed statistical tests, number of 'n' in each experiment]; keywords on first page of manuscript). Attached are the completed manuscript file, the updated figures, the updated extended view figures and the updated appendix.

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Prof. Dr. Hellmut G. Augustin	
Journal Submitted to: Embo Mol Med	
Manuscript Number: EMM-2016-07222	

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
➔ definitions of statistical methods and measures:

- common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
- are tests one-sided or two-sided?
- are there adjustments for multiple comparisons?
- exact statistical test results, e.g., P values = x but not P values < x;
- definition of 'center values' as median or average;
- definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	n>=3 (cell experiments) n>=5 (animal experiments) n>=10 (human tissue samples)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n>=5 TTEST
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	sick animals were excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	na
For animal studies, include a statement about randomization even if no randomization was used.	each animal was refered to with a number
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	blinding of investigator; validation of results by two independent investigators

4.b. For animal studies, include a statement about blinding even if no blinding was done	validation of results by two independent investigators. Each animal was refered to with a number.
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes, support by statistician
Is there an estimate of variation within each group of data?	yes
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Endosialin antibody: MacFadyen et al, 2005. All other antibodies: Mogler et al., 2015.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were tested neg for mycoplasma by PCR. LX-2 cells purchased from Millipore. Huh7 cells kindly provided from PD Dr. Breuhahn (Institute of Pathology, Heidelberg)

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse, C57/BL6, male, 8-12 weeks for experiment initiation, breeding into AST Cre mouse model (Runge et al 2014 Cancer Research). Endosialin knockout (Mogler et al., 2015). Animals were housed in barriers at the animal facility of the DKFZ with free admission to food and water.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed according to the guidelines of the local Animal Use and Care Committees and approved by the Regierungspräsidium in Karlsruhe (35-9185.81/G-228/10).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	compliance confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Study approved by the Ethics Committee of the Heidelberg University (Ethical votes # 206/207, 2005).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	This study was registered at the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) and performed according to the Declaration of Helsinki; written informed consent was obtained from all patients. All patient specimen were exclusively provided in a ~~pseudonymized form according to the Standard Operating Procedures of the NCT~~
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	na
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	na
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	na
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	na
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	na

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	na
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Figure EV10
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	na

21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	na
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	na

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	na