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Obesity and diabetes accelerate hepatocarcinogenesis via hepatocyte proliferation independent of NF-κB or Akt/mTORC1

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Handling editor:
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Date: 4-Jan-2016

Ref.: Ms. No. JCTRes-D-15-00016
Obesity and diabetes accelerate hepatocarcinogenesis via hepatocyte proliferation independent of NF-κB or Akt/mTORC1
Journal of Clinical and Translational Research

Dear Ms. Arfianti,

Your paper has been reviewed by an external referee and the editorial board. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' and editorial comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Your revision is due by Jan 20, 2016.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Rowan van Golen
Associate Editor
Journal of Clinical and Translational Research

*****Reviewer comments*****

Reviewer #1: The current manuscript describes that obesity induced via fox/foz mutation increases HCC development. The authors claim that the increased hepatocarcinogenesis is not caused by accelerated TNF/NFkB, IL-6 stat3 and IGF1/akt/mTor but instead is caused by increased dan damage response. moreover, the authors aim at elucidating the mechanisms that precede hepatocarcinogenesis by using mice at 3, 6 and 9 months of age.
The manuscript is well written, however owing to the following concerns I propose to reject the manuscript with the possibility for resubmission.
1. the data sets are incomplete. authors show datasets from 3 and 6, then data from 3 and 6 months only without any explanation. I m sure the authors have the missing data sets, they have to be included at least for supplemental information.
2. the authors compare their data to the current literature. however, the injected 10mg DEN/kg BW instead of commonly used 25 mg/kg BW. why is this so? a reasonable explanation should be given and this point has to be discussed. Further a quantification of timor load should be included at the indicated time points.
3. in the not well conducted/shown experiments figure 5 A-F the authors aim at disproving the work from numerous labs that clearly have given novel molecular insights into how TNF and IL-6 contribute to hepatocarcinogenesis. they expect TNF is high on RNA level and increasingly activates NFkB. Instead it is the other way around. inhibition of NFkB increases liver tumorigenesis as shown by several papers of the karin lab using IKKbeta KO mice or pasparakis lab using hepatic nemo ko mice. Further, in the absence of hepatic nemo also obesity increased hepatocarcinogenesis (Wunderlich et al., 2008 PNAS). A quantification of nuclear p65 from a western blot with unequal loading is clearly insufficient to disprove such data. On the other hand, it has been proposed that TNF induced JNK accelerates HCC which has not been addressed at all. Furthermore, IL-6 induced stat3 axis is inadequately addressed. i) serum IL-6 levels at 6 months do not differ between wt and foz mice, what about RNA level also at the other time points? according to the western blot 5F (which is not described in the manuscript), there is more P stat-3 in den induced foz and HCC which is not represented in the bar chart of 5D. Why is 5E Mcp-1 RNA included without any explanation? In my opinion, the data to exclude TNF and IL-6 action in obesity associated HCC are inadequate.
4. Fig 6 addresses insulin IGF axis. only one comment: why are foz mice insulin resistant but show increased activation of downstream mediators such as P-AKT? is insulin resistance not causing the opposite?
5. rapamycin treatment experiments in fig. 7 and 8 were not done (or not shown) for wt mice.

Further there are some minor points such as:
Fig. 2A, B are not described in the text.
in the introduction authors state that IL-6Ralpha knock down instead of knock out
Fig 1B relative fat mass? ewat/bw ratio? BW was shown already in fig. 1A. why not showing swat weight? or nor data on fat content?
why is there not a single sentence that describes the nature of the foz mutation? could this be the hint?
Collectively, I think the data as they are presented cannot support the conclusion drawn by the authors and thus, the manuscript in its present form is too premature to be published.

#2 Editorial comments:
Given the good fit with the JCTR scope and the (potential) scientific impact of the manuscript, the editorial board sees merit in your work. We would therefore like to ask you to carefully prepare a rebuttal to the points raised by reviewer #1. In particular, the presented data should be more comprehensively discussed in context of current literature and potential technical errors such as unequal WB loading should be elucidated.

Authors' rebuttal:

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Canberra, 17 January 2016

Re: revision JCTR-D-15-00016: Obesity and diabetes accelerate hepatocarcinogenesis via hepatocyte proliferation independent of NF-□B or Akt/mTORC1

Dear Dr van Golen,

Thank you for your helpful letter of 5 January 2016 concerning the above manuscript, and for offering us the opportunity to submit a revised version to Journal of Clinical and Translational Research

We have addressed the constructive comments of the reviewers (attached as supplementary material, not for publication). In the attached document, we provide a detailed response to reviewer comments *in red italics*, as required.

We appreciate the thoughtful comments of the reviewers in pointing out potential limitations of the data and their interpretation, as well as a need to improve the quality of some figures. As a result of responding to these concerns, we believe the manuscript has been appreciably improved.

We hope that our responses and the manuscript revision will bring the article to the high scientific standards required for publication in JCTR.

On behalf of the authors

Yours Sincerely

Geoffrey Farrell

Reviewer #1:

The current manuscript describes that obesity induced via foz/foz mutation increases HCC development. The authors claim that the increased hepatocarcinogenesis is not caused by accelerated TNF/NFκB, IL-6 stat3 and IGF1/akt/mTor but instead is caused by increased DNA damage response. Moreover, the authors aim at elucidating the mechanisms that precede hepatocarcinogenesis by using mice at 3, 6 and 9 months of age. The manuscript is well written, however owing to the following concerns I propose to reject the manuscript with the possibility for resubmission.

We appreciate the view that our manuscript is well written.

The data sets are incomplete. Authors show datasets from 3 and 6, then data from 3 and 6 months only without any explanation. I am sure the authors have the missing data sets, they have to be included at least for supplemental information.

With respect, the reviewer may not have fully appreciated that our intent was to establish the molecular signalling pathways by which obesity accelerates HCC onset. This was stated in para 3 (at end) and para 4 of the introduction [highlighted in green], and even more clearly at end of para 3.1 of results (viz: "Since the focus of the present studies was ***on the molecular events that precede onset of obesity-related HCC****, subsequent measurements were performed on tissues harvested at 3 and/or 6 months".*

We see no value on providing a set of these readouts at 9 months, when ***both lean and obese mice have DEN-induced HCC****. In order to provide the reader with clearer guidance as to the unique purpose of our studies, we have clarified this in the ABSTRACT (see revised Aims) "we tested the proposed involvement of NF-□B, IL-6/STAT3 and Akt/mTORC1 before onset (at 3 months) and at onset (6 months) of accelerated hepatocarcinogenesis in DEN-injected obese and diabetic* foz/foz *compared to lean wildtype mice, and also studied the hepatocyte proliferative response to DNA damage between the obese and lean lines."*

The authors compare their data to the current literature. However, they injected 10mg DEN/kg BW instead of commonly used 25 mg/kg BW. Why is this so? A reasonable explanation should be given and this point has to be discussed.

Thank you for pointing out the differences in DEN dose used in hepatocarcinogenesis studies over the last 30 years. As an historical reference point, Drinkwater N and colleagues (Moore et al, 1981) found that 0.1 µmol/L/g body weight, which equates to 10 mg/kg, was sufficient to

cause HCC in male mice. Our philosophy has been to use the minimal carcinogen dose that reliably causes liver cancer in lean male mice at 9 months of age, which as evident from Table 1 is highly reproducible (~100%) in our hands (and see refs 18 and 24) at this dose or even lower (5 mg/kg).

We see no virtue in using a more hepatotoxic dose (like 25 mg/kg) of DEN because the intent of the model is to be as close as possible to the usual human context of spontaneous HCC in NASH or cirrhosis. Occupational health hazards aside (DEN is a powerful carcinogen), higher DEN doses cause more liver injury, with resultant more liver inflammation – and this is a potential explanation for why some (not all) other workers have found NF-□B activation and IL-6 release in DEN-injected mice, including those fed a high fat diet which would further exacerbate liver injury. It is unclear whether other workers used the higher dose because they failed to obtain liver cancers at the lower dose (we are aware of many instances where this has been the case because of flawed technical details, like the precise day of injection, making up the reagent fresh and keeping it from light exposure). The fact that in one article cited by the reviewer (Maeda et al, 2005) actually used TWO (2) different doses of DEN (respectively 5 mg/kg and 25 mg/kg) gives us pause to consider this could have been the case.

Finally, we note that the following articles have used exposure of 12-15 day old mice to 10 mg/kg body weight of DEN to achieve a highly efficient (90% or more) induction of liver tumours at 9 months (Bopp et al, 2015; Hatayama et al, 1993; Kuklin et al, 2004; Rignall et al, 2011; Vesselinovitch, 1990). There are fewer articles using the higher dose (25 mg/kg body weight, even 80 mg/kg body weight), and it is our view that it would be more helpful if those authors indicated why that was the case, rather than ourselves having to justify a less toxic, highly reproducible regimen.

Further a quantification of tumor load should be included at the indicated time points.

At 3 month, neither foz/foz nor Wt mice developed any macroscopic liver nodules. All DEN-injected foz/foz mice developed large (some truly massive, see Fig. 2A) liver tumour nodules at 6 months, and the 4 (only) 11 Wt counterparts developed 1 or 2 pin point nodules. Thus, persusal of figure 1C indicates no increase in relative liver mass in Wt mice at 6 mo that could be attributable to tumors, whereas there is a highly significant and rather large further increase in DEN-injected foz/foz mice, which reflects substantive tumor bulk. With this perspective we did not see the need to dissect out all liver tumors for a separate quantification of tumour load. A comment about the inference of hepatomegaly for tumor load has been added to the results section 3.1.

In the not well conducted/shown experiments figure 5 A-F the authors aim at disproving the work from numerous labs that clearly have given novel molecular insights into how TNF and IL-6 contribute to hepatocarcinogenesis.

Apart from research from the Karin lab, most work about IL-6 and HCC comes from clinical correlations and is not informative as to possible mechanisms. Further, the specific purpose of

our research was to address only mechanisms pertinent to obesity and diabetes-related acceleration of HCC, and in the literature we searched we found only the Park et al article [ref 4] has addressed this point. As mentioned earlier, these investigators used 25 mg/kg body wt of DEN and this provides a partial explanation for differences between our 2 studies. Of interest to the point of consistency, the same lab has recently published 2 major articles on mTORC1 and HCC. In JCI 2014 (Inokuchi-Shimizu et al, 2014) they conclude (last sentence of abstract): "These data indicate that TAK1 regulates hepatic lipid metabolism and tumorigenesis via the AMPK/mTORC1 axis, affecting both autophagy and PPARa activity". In the second (preceding month in Cell Metab 2014 (Umemura et al, 2014) they showed that rapamycin treatment (to block mTORC1) enhanced HCC development! We trust that or own very thorough research at least has the virtue of reproducible conclusions.

They expect TNF is high on RNA level and increasingly activates NFkB.

No, our point is that there is no increase in serum TNFa in DEN-injected obese versus lean mice. We have now added the serum TNF data (see Figure 5A) (values are largely undetectable as we understand other labs have found), and modified Figure 5 to include hepatic TNF protein data (Figure 5C). The results show that TNFα was even lower in livers of saline-injected obese foz/foz than Wt littermates at 3 mths, while there were no significant changes in other groups at both 3 and 6 mths. The point is that p65 expression is not increased in this work, differing from the inference by Park et al (they didn't actually show hepatic NF-kB expression) that TNF (via TNF-R1) is what is causing IL-6 release in their high DEN, high fat diet model.

Instead it is the other way around. Inhibition of NFkB increases liver tumorigenesis as shown by several papers of the karin lab using IKKbeta KO mice or pasparakis lab using hepatic nemo ko mice. Further, in the absence of hepatic nemo also obesity increased hepatocarcinogenesis (Wunderlich et al., 2008 PNAS).

The latter paper did not use DEN or obesity (the authors used a high fat diet – this is not the same thing as obesity with metabolic syndrome and diabetes.)

A quantification of nuclear p65 from a western blot with unequal loading is clearly insufficient to disprove such data.

The reviewer may not have appreciated that the data in Fig. 5(D,G) (see revised manuscript) are generated from 10 mice (see last phrase of figure legend), not on the few sample WB ill-advisably chosen for illustration in Fig. 5(G). We apologise for the variability shown in the WB data, and have now replaced Fig. 5(G) with more representative illustrations.

On the other hand, it has been proposed that TNF induced JNK accelerates HCC which has not been addressed at all.

The reviewer raises an interesting point. JNK can also be activated by reactive oxygen species (ROS), free fatty acids, free cholesterol and insulin, some or all of which are likely to be

important in our model. We intend to pursue this promising direction in future studies using Jnk1$^{-/-}$ and Jnk2$^{-/-}$ mice, but this would comprise a major study in itself.

Furthermore, IL-6 induced stat3 axis is inadequately addressed. i) serum IL-6 levels at 6 months do not differ between wt and foz mice, what about RNA level also at the other time points? According to the western blot 5F (which is not described in the manuscript) [*corrected – thankyou*], there is more P stat-3 in den induced foz and HCC which is not represented in the bar chart of 5D [*no, the review again extrapolates from one of 10 blots, which have been correctly averaged as a large group composite in Fig. 5(F). Figure 5(G) has been made more representative of these blots*].

Why is 5E Mcp-1 RNA included without any explanation? [*this was an oversight and it has now been omitted*]

In my opinion, the data to exclude TNF and IL-6 action in obesity associated HCC are inadequate.

In the present work, serum IL-6 levels were noted to be low and comparable between obese foz/foz and lean Wt mice (Fig. 5E). However, we are concerned about development of dogma in the field of obesity-related HCC (which is why we conducted these thorough studies with a more relevant model and a more relevant dose of DEN). Further, we are not alone! Several authors have found that ob/ob mice fail to display high circulating levels of IL-6 (Fantuzzi & Faggioni, 2000; Griffin et al, 2009), despite enhanced DEN-induced HCC development (Park et al, 2010). We conclude that while the relationship between IL-6 and obesity-related hepatocarcinogenesis remains unresolved to the satisfaction of all investigators, it may not be applicable to all models of liver cancer and all models of obesity. We hope that those interested in the field will find the present data interesting as a counter point to those of the Karin lab, and in accord with work in ob/ob mice.

Fig 6 addresses insulin IGF axis. only one comment: why are foz mice insulin resistant but show increased activation of downstream mediators such as P-AKT? is insulin resistance not causing the opposite?

This is a good point. Since what we measured (strictly speaking) was serum insulin concentration and not insulin resistance, we have modified the text to specify that and remove the apparently conflicting reference to "insulin resistance". We appreciate the concept of "partial insulin resistance" (ie impairment of some but not all arms of insulin receptor signalling is not always appreciated eg failure of insulin to suppress hepatic glucose output but at the same time stimulating hepatic lipid synthesis via SREBP1) is insufficiently well known to use it loosely.

Rapamycin treatment experiments in fig. 7 and 8 were not done (or not shown) for wt mice.

We fail to appreciate the reviewer's point. The rapamycin intervention was to test whether the mTOR activation observed in foz/foz (but NOT in Wt mice!) was involved in early-onset HCC (which did not occur in lean Wt mice). After all, this is what the Karin group contended in their JCI 2014 article (and disproved in the Cell Metab article published a month apart in 2014). There was no point in scientific purpose or experimental design to include a rapamycin experiment on Wt mice, and it would have been irresponsible (from animal ethics viewpoint) to conduct such a meaningless experimental arm.

Fig. 2A, B are not described in the text.

We apologise for this inconvenience. We have elaborated more about Fig 2A,B in the text (see para 1 section 3.1).

In the introduction authors state that IL-6Ralpha knock down instead of knock out

We apologise for this mistake. Changed accordingly, thank you.

Fig 1B relative fat mass? epiWAT/BW ratio? BW was shown already in fig. 1A. Why not showing as epiWAT weight? or nor data on fat content?

We think relative fat content (relative to BW) is more informative than epiWAT weight in comparing body composition between the 2 lines. Naturally, this underestimates differences, but the interested reader can use the data in Fig. 1(A) to make adjustments to absolute values if they chose.

Why is there not a single sentence that describes the nature of the foz mutation? Could this be the hint? [*Hint to what? Meaning is unclear – sorry. There are actually 4 refs [refs 19-22] in the article. The underlying Alms1 mutation that determines appetite dysregulation is detailed in them and cross references to them (there are now nearly 20 articles using these mice in metabolic studies).*

#2 Editorial comments:

Given the good fit with the JCTR scope and the (potential) scientific impact of the manuscript, the editorial board sees merit in your work. We would therefore like to ask you to carefully prepare a rebuttal to the points raised by reviewer #1.

This has been done –see above comments for reviewer #1.

In particular, the presented data should be more comprehensively discussed in context of current literature and potential technical errors such as unequal WB loading should be elucidated.

Thank you – this has been done – see (Figure 5G in the revised manuscript).

References

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2nd editorial decision:

Date: 19-Jan-2016

Ref.: Ms. No. JCTRes-D-15-00016R1
Obesity and diabetes accelerate hepatocarcinogenesis via hepatocyte proliferation independent of NF-κB or Akt/mTORC1
Journal of Clinical and Translational Research

Dear Ms. Arfianti,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

Comments from the editor and reviewers can be found below.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers:

Dear authors,

Based on the modifications implemented in the manuscript as well as the rebuttal provided the editorial board has decided to accept your manuscript for publication. The changes that were made resulted in an improved quality of the work and yielded further credence to the conclusions. The changes were made in accordance with the comments of the reviewer, who is a prominent expert in this field. You have also clearly indicated why some changes were not enforced, citing important literature to corroborate your standpoint. Also, a clear rationale was provided for the choice of animal model in combination with the dosage used to induce carcinogenesis, all in line with JCTR's philosophy.

Congratulations with this accomplishment.

On behalf of the JCTR editorial board,

Michal.