Format for ANSWERING REVIEWERS

December 15, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 14947-edited.doc).

Title: Autophagy in anti-apoptotic effect of augmenter of liver regeneration on HepG2 cells

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 14947

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

(1) 01568246:

1) The molecular mechanisms whereby ALR increase autophagy and reduce apoptosis are still unknown. The authors should discuss/suggest possible explanations. Revision has been made according to the suggestions of the reviewer. The possible mechanism is that ALR may promote autophagy by inhibition of cleavage of Beclin-1, which can be mediated by Caspase-3. As far as we known, ALR may inhibit the activation of Caspase-3 through promoting ATP synthesis and reducing the release of cytochrome c.

2) When using accumulation of LC3-II to measure autophagy it is necessary to distinguish whether autophagosome accumulation is due to autophagy induction or rather a block in downstream steps, for instance reduced fusion of autophagosomes/amphisomes with lysosomes. One possibility to measure the real autophagic activity would be to inhibit degradation in lysosomes (autolysosomes). This could be done by means of protease inhibitors such as leupeptin (used by Seglen and coworkers, Autophagy 3:3 181-206 2007). The actual increase in autophagic activity would be the difference in accumulation with or without the inhibitor. This problem is discussed in one of the papers referred to in the present manuscript (ref 12).Could I resolve this problem by measuring p62 which is a substrate for lysosome? If I get the reduction of p62 after transfection of ALR plasmid, it will mean autophagosome accumulation is due to autophagy induction rather than a block in downstream steps. In our study, the levels of p62 were lower in HepG2 cells treated with ALR than control (Fig 2A, $P<0.05$), which suggested that ALR treatment did not block the fusion of autophagosomes with lysosomes.

3) A main problem with the paper is the language. In several places in the article it is difficult and even not possible to understand what the authors mean. Some examples: In Abstract (Background): (a) The sentence starting with: "Multiple evidences.." is not clear and should be rewritten. It should give real information about the processes. What are the mechanistic overlap and the interaction between the apoptotic machinery and autophagy proteins? Maybe this information could be given in the Introduction. (b) The sentence starting with "HepG2 cells were treated by.." should be rewritten. The cells were not treated by inhibition of autophagy, and "to observe" should be deleted and the rest of the sentence should read: Apoptosis were observed by fluorescence microscopy and flow cytometry". (3) "The counts of apoptotic cells were much more…" should be replaced with "The counts (number) of apoptotic cells were much higher…" In Introduction: (1) The first sentence in Introduction is not clear: What can stimulate DNA synthesis? HSS or partial hepatectomy? The sentence should be rewritten! (2) The second paragraph (section) in the Introduction is very muddled and should be abbreviated and give a clearer overview of what is known about the relation between autophagy and apoptosis. In Results: (1) In second

paragraph: ”As well as we know” change to: “As far as we know”. (2) In “ALR increased autophagic activity in HepG2 cells”: “After starving for 24 hours…green puncta in HepG2 cells treated with ALR were more than that in control” change to “….green puncta in HepG2 cells treated with ALR were more numerous than in control cells”. Later in same paragraph: “the number of autophagosomes…..was more than that in control”, change to “was higher than that in control”. (3) In “3MA suppressed autophagic activity in HepG2 cells”. The first sentence is difficult to understand. What is meant with “to inhibit autophagic formation for the importance of Beclin 1/class-III PI3 kinase complex”? Rewriting. All the sentences were rewritten according the suggestion of the reviewer.

(2)01166697

1) ALR should be better explained in the Introduction. In the Introduction section, the mechanisms of autophagy and apoptosis are confusing and their overlap is not clear neither is the role of ALR. Revision has been made according to the suggestions of the reviewer in the introduction. The overlap of autophagy and apoptosis is very clear as followed as Fig2.

2) In the Abstract, Background section, where is the verb for the sentence: “Multiple evidences suggested that mechanistic overlap and interaction between the apoptosis machinery and autophagy proteins”? The verb was added.

3) In the Introduction section, which is the reference number in the sentence: “Polimeno et al. demonstrated that ALR can….”? The reference number was added.

4) Which is the reference for those two plasmids: GFP-LC3B and ALR. The references were added.

5) In the Material and methods’ section the authors claimed that: “For quantification of autophagic cells, GFP-LC3 puncta were determined from triplicates by counting a total of more than 30 cells”. The number of counted cells is quite small, the authors should increase such number. The same is for electron microscopy evaluation. The reference number was added.

6) In the figure legend, Fig 1, the authors claim that “the number of typical autophagosome…”, however, they did not show any graphs. What is such number? The number was deleted.

7) In Fig 2, it is not clarified which sample is transfected with control and with LC3 plasmid, making the comprehension of the figure very difficult. For instance, what are the four lanes of the western blot in Fig 2A? For western blot and electron microscopy, we did not need to transfect GFP-LC3 plasmid but ALR plasmid to HepG2 cells. Only for fluorescence microscopy, we needed to transfect both GFP-LC3 and ALR plasmid and GFP-LC3 was as an autophagic marker (green dot).

8) To claim that: “All results indicated that ALR increased autophagic flux in HepG2 cells…”, in pag 6, the authors should perform also western blot of the p62 protein, as read-out of the autophagic flux. The western blot of the P62 protein was performed.

9) In Fig 3, again, what are the samples in the western blot? The figure legend is very confusing. The answer was same as 7).

10) In the Discussion section the authors claim: “Based upon these premises, we verified the hypothesis of an involvement of autophagy in the anti-apoptosis effect of ALR on hepatocytes”, however, they used a hepatocarcinoma cell line, instead of hepatocyrs. Please explain this discrepancy. I am sorry for this discrepancy. I have used the liver instead of hepatocytes already.

(3)00069015

1) Why only one cell line, HepG2 was used? One would wonder what the effects in other hepatoma cancer cells. I also used other cell line such as hul7 to do the same experiments and got the same results.

2) Discussion includes a careful description of the experimental data but lacks the discussion of the global meaning of the results. The comparison of the findings with other scientific reports, as well as a final sentence on the future perspectives of the research are absolutely required. Conclusions needs to be extended with a more general statement. I tried to make a revision according to the suggestions of the reviewer in the discussion. If possible, please give me more specific suggestion. Thank you very much!

3) English needs to be edited in the whole manuscript and checked for typos. I will seek to make use of a copyediting service provided by professional English language editing companies.

(4) 00004603

1) You made a conclusion that ALR activates authophagy based on induction of LC3-II. However, LC3 in autophagosome indicates only upstream level of autophagy induction, which may be also related to impairment of lysosomal function. To show that there are no changes in lysosomal degradation, you need at least to measure p62, which is a substrate for lysosome. If you get an accumulation of p62 after transfection of ALR plasmid, it will mean that ALR suppresses lysosomal degradation and it will indicate the reduction in autophagy even LC3 levels go up. Yes, I measured p62 level after transfection of ALR plasmid in HepG2 cells.

2) LC3 induction by itself cannot indicate autophagic flux. Yes, I measured p62 level after transfection of ALR plasmid in HepG2 cells.

3) You need to provide the data on efficiency of plasmid transfection. There is a picture to show the efficiency of plasmid transfection as followed as Fig1.

4) There are lots of misspelings in the text. Yes, I will seek to make use of a copyediting service provided by professional English language editing companies.

(5)01566894

1) This is a poorly written paper with numerous grammatical and spelling errors. Yes, I will seek to make use of a copyediting service provided by professional English language editing companies.

2) Authors' conclusion is quite contradictory to the data presented, and could be misleading.

3) Autophagy is highly dynamic and the changes in static levels of LC3 do not reflect this important feature of autophagy. Although authors mentioned the autophagic flux on page 5, its validity and interpretation are questionable. Yes, I measured p62 level after transfection of ALR plasmid in HepG2 cells.

4) All assays were performed in combination with both ALR and starvation. ALR alone should be assessed. If I did not remove serum from HepG2 cells, autophagy would not be induced and the effect of ALR on autophagy would not be assessed.

5) The concentration of 3-MA used in this study is microM, which is not enough to block autophagy. The concentration of 3-MA can be found in reference 10.

6. The interpretation of Fig. 4 is not appropriate since fluorescence imaging of Annexin-V and PI does not support apoptosis.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

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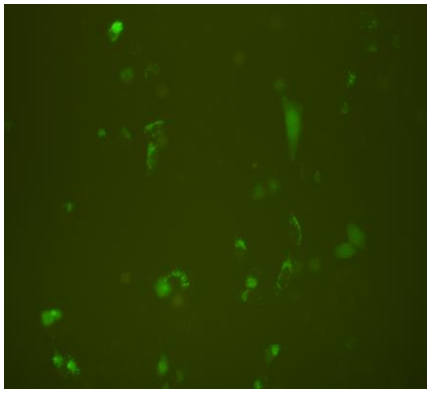

Fig1 The green cells were GFP positive cells which indicated that the transfection succeeded.

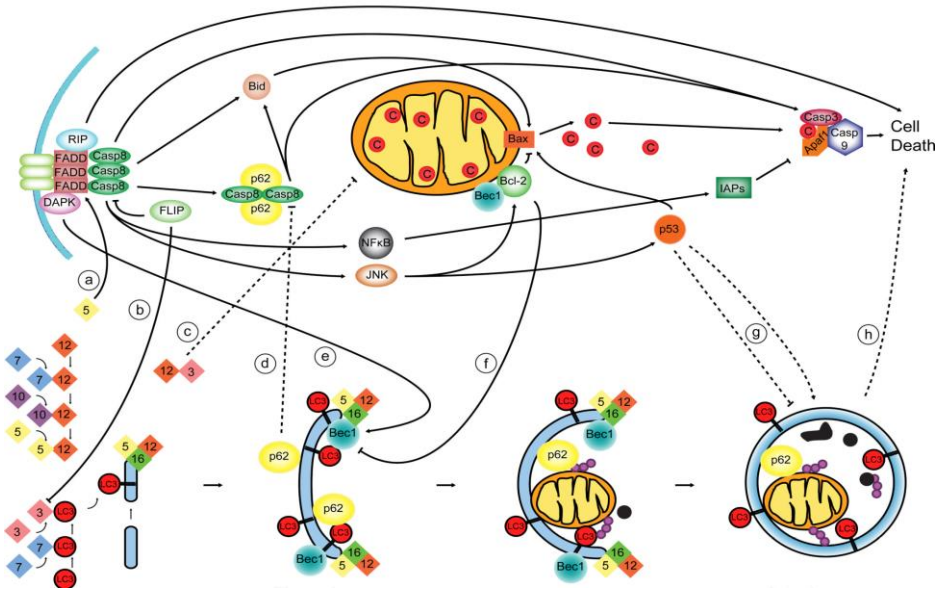

Fig2 The correlation between apoptosis and autophagy. (Gump JM, Thorburn A. Autophagy and apoptosis: what is the connection? Trends Cell Biol. 2011; 21(7): 387-392.)