Manuscript NO: 52092

Title: LB100 ameliorates nonalcoholic fatty liver disease via the *AMPK / Sirt1* pathway

Dear Prof. Tang,

Thank you very much for your email dated 5 November 2019 and the valuable comments of the four reviewers. We have revised the manuscript, and would like to re-submit it for your consideration. We have addressed the comments raised by the reviewers, and the amendments are highlighted in yellow in the revised manuscript. Point by point responses to the reviewers' comments are listed below this letter. We hope that the revised manuscript is acceptable for publication. Thank you!

Yours Sincerely,

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Replies to Reviewer #1:

Comment 1: Authors are kindly requested to compare their results with those below presented: SIRT4 could exert its tumor suppressive function in HCC by inhibiting glutamine metabolism and thereby increasing the adenosine diphosphate (ADP)/AMP levels to phosphorylate AMPKα by LKB1, which blocks the mTOR signaling pathway, as ev ident in HepatologyVolume 69, Issue 4 Sirtuin 4 Depletion Promotes Hepatocellular Carcinoma Tumorigenesis Through Regulating Adenosine-Monophosphate–Activated Protein..... and..... Oxid Med Cell Longev. 2014;2014:920676. doi: 10.1155/2014/920676. Epub 2014 Jun 17. Circulating levels of sirtuin 4, a potential marker of oxidative metabolism, related to coronary artery disease in obese patients suffering from NAFLD, with normal or slightly increased liver enzymes

Reply: Thank you very much for your valuable advice!

In Tang's research，depletion of SIRT4 decreased the expression of p-AMPKα and activation of AMPKα by metformin fully reversed the oncogenic features in SIRT4-negative cells ,which indicated that SIRT4 is the upstream of AMPKα and has a positive regulation in tumorigenesis. While in our research, there is no obvious date to prove the upstream and downstream relationship between the two molecules. Just like other research showed, AMPK and Sirt1 are activated each other in a finely tuned network[1]. In my opinion, although SIRT1 and SIRT4, both having a conserved deacetylase domain, are members of the

seven sirtuins in mammals. They possibly have different roles in regulating tumorigenesis and lipid metabolism. Besides, Tang didn't demonstrate any effect to SIRT4 when inhibiting the phosphorylation of AMPK. So more research may be required to dig out the complex relationship between sirtuins and AMPK in tumorigenesis and lipogenesis.

In Giovanni Tarantino's cross-sectional study, the serum levels of SIRT4 were present significantly decrease in obese patients compared to the healthy subjects, while those with severe grade of hepatic steatosis have lowest serum SIRT4 concentrations. The result predicted hepatic steatosis possibly is the reason of low serum levels of SIRT4. Consistent with our study, sirtuins, such as SIRT1, regulate lipid homeostasis through adipogenic genes such as SREBP1, FAS, ACC and SCD1 and increased the expression of fatty acid β-oxidation genes to inhibit the development of NAFLD. However, one limitation of our study is lack of clinic data. While Giovanni Tarantino's research is a appropriate complement to our research. The study also gives us a hint to detect the concentration of SIRT1 in the serum of NAFLD patients, so that expand the clinical significance of our research.

Replies to Reviewer #2:

Comment 1: previous studies showed that the well-known medication for example Metformin may have a role on Sirt1 and AMPK activations, thus the authors should clarify what's new of the efficacy of LB-100 in comparison to MFM on Sirt1 and AMPK pathways.

Reply: Thank you for this comment!

Numerous studies have found that AMPK and Sirt1 are closely related to lipid metabolism and activate each other in a finely tuned network. Currently, there are a few studies mentioned about the role of AMPK / Sirt1 pathway and therapeutic potentials in NAFLD for example metformin. There are some difference between metformin and LB100. First, metformin activates AMPK indirectly by inhibiting Complex I in the respiratory chain, but PP2A regulates the phosphorylation of AMPK, the direct interaction between PP2A and AMPK was confirmed [2, 3].Besides, in many studies, the dosage of metformin varies from 50-300 mg/kg/d in mice, and 0.1-0.4 mM in vitro. While in our study, we injected mice with LB100 at a dose of 1.5 mg/kg (three times a week), and in vitro, L02 cells were exposed to only 6 μM LB100. In addition, metformin treatment may lead to occurrence of lactic acidosis, as for LB100, is currently in two clinical trials (NCI designated NCT03027388 and NCT01837667). A phase-1 safety study with 7 dose-escalations did not note relevant toxicity[4].

Comment 2: Autophagy which played an important role in lipid catabolism (lipophagy). Previous study showed that the elevated levels of methionine and SAMe activated PP2A by methylation. Can the author link the association of your results with previous study?

Reply: Thank you very much for your valuable advice!

PP2A plays multiple roles in different signaling pathways and regulates diverse cellular processes such as transcription, intermediary metabolism, apoptosis and autophagy. Results of the present study show that PP2A have essential roles in the regulation of autophagy. Marta Varela-Rey's study showed blocking PP2A activity restored autophagy flux in hepatocytes and ameliorated liver steatosis[5]. While some studies have described that inhibition of PP2A by okadaic acid suppresses autophagy in hepatocytes[6, 7]. Our study in which LB100 works through the activation of AMPK/Sirt1 pathway adds an exciting new mechanism through which LB100 exerts its beneficial effects on ameliorating lipid accumulation. Undoubtedly, it is interesting to perform further studies to establish whether LB100 ameliorates liver steatosis through the regulation of autophagy.

These opinions are added in our DISCUSSION part and we have highlighted this content. (Page36 ; Line7-15)

Replies to Reviewer #3:

Comment: In this well designed study, authors demonstrated that LB100 can play an important role in liver lipid metabolism to prevent HFD-induced obesity, hepatic steatosis and IR in a NAFLD mouse model. LB100 reduced hepatic lipogenesis and promoted fatty acid β-oxidation via the AMPK/Sirt1 pathway in HFD-fed mice. These findings provide compelling evidence supporting LB100 as a promising therapeutic for NAFLD.

Reply: Thanks for the reviewer's positive comments!

Replies to Reviewer #4:

Comment 1: As a clinician, I need the data on liver histology including quantification of fibrosis at the end of experiment.

Reply: Thank you for this comment!

NAFLD ranges from simple steatosis to NASH, irreversible fibrosis, cirrhosis, and eventually HCC. High fat diet(HFD) has been widely used for decades to induce obesity and insulin resistance in mice. A model of a HFD in male C57BL/6 mice led to the development of features of the metabolic syndrome and steatohepatitis but only mild fibrosis after 50 weeks[8].In our study, mice were fed HFD for 16 weeks, thus it hardly to develop liver fibrosis. We demonstrated liver fibrosis by both the staining of Sirius red and Masson's

trichrome, and conformed that there were almost no signs of fibrosis. We added this results as **Fig. S4** and have highlighted this content.

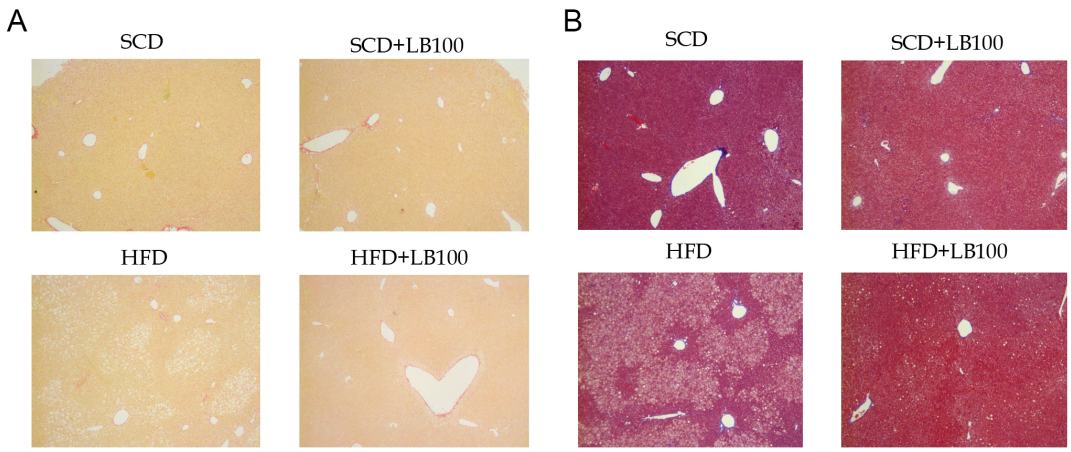

Fig. S4. Liver fibrosis was evaluated by staining of Masson's trichrome and Sirius red. A: Representative liver histological section images of the four groups of mice stained with Masson and B: Sirius red.

Comment 2: Quantification of fatty acid in liver cell is the primary insult in the NAFLD pathway.

Reply: Thank you for this suggestion!

You are right that we didn't measure hepatic and cellular fatty acid levels. Actually, measurement of fatty acid concentrations in tissues does not provide information about the net flux through the system. Studies have confirmed that the intracellular fatty acid concentration is fairly constant in the liver, suggesting that elevations of fatty acid concentrations in hepatocytes are unlikely to be associated with lipotoxicity[9]. Fatty acid disposal in the liver

occurs through the formation of triglyceride (TG), which is then stored as lipid droplets, resulting in hepatic steatosis. TG accumulation is a hallmark of NAFLD. In our study, we tested hepatic and intracellular TG content to evaluate levels of lipid accumulation, and this may be reasonable.

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