Dear editor and reviewers:

Thanks a lot for your letter and reviewers' comments for our manuscript entitled "The maturity of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS)-derived liver regeneration in a rat model (Manuscript ID 37663, World Journal of Gastroenterology)". These instructions are valuable and very helpful for revising and improving our paper. They make us think through our work and get enlightened for some new ideas as well. We have studied comments carefully, made corrections and added explanations that we hope meet with approval. Revised portions are marked in blue or red in the paper.

Review#1's first question regarding that "The authors have not used the 70% standard of hepatectomy (in fact, they do not reflect what percentage of liver they remove at the end, which they should do to give it some scientific rigor.) In 5 rats they only remove the LLL which logically induces less regeneration, since only 35% of the total hepatic volume is removed, and in the remaining 5 rats, 80% die."

Response: Sincerely thanks for raising the issue.

Firstly, we are very sorry for that the results in the preliminary study were not stated clearly. In fifth day after operation, the ratio of right middle lobe to body weight (RML/BW) were 1.48% in the LLL group and 1.10% in the sham group, respectively ($p<0.001$). For the extended PHx group (removal of left lateral, left middle, right and caudate lobes), the mortality was 80%. And in this group, the RML/BW of survival rat (n=1) was 3.44%. Therefore, removal the LLL could induces less regeneration logically, which is consistent with the literature report.[1-2]

Meanwhile, considering the feasibility and proliferative capacity of PHx models with different extents of hepatectomy, the medium PHx group (removal of left lateral, right and caudate lobes) was established in this study. In this model, the liver regeneration could be triggered rapidly and the mortality was only 20% (Supplement figure 1). Therefore, such model was regarded as the positive control in our study.

Review#1's second question regarding that "The ALPPS technique in rats should simulate

the surgical technique that is performed in humans, so they should also ligate the portal branch of the left middle lobe of the rat."

Response: Thanks for this good question that helps us improve our paper.

We are very agreeing with review's opinion that the ALPPS technique in rats should simulate the surgical technique that is performed in humans. However, the liver anatomy is totally different between rat and human beings. The liver of rat contains five parts including left lateral lobe (LLL), caudate lobe (CL), right lobe (RL), left middle lobe (LML) and right middle lobe (RML). Meanwhile, the LML and LLL have the common trunks. To induce a feasible ALPPS model in rat or mouse, two types of model (in rat and mouse) have been established.[1-4] One is resection of LLL+ ligating the portal vein of CL+RL+LML; the other one is ligating the portal vein of CL+RL+LML+LLL. Both models should transect the liver parenchyma between LML and RML. In our study, we chose the latter one (ligating the portal vein belonging to CL+RL+LML+LLL, but without resection of any lobe, Figure 1A and Supplement figure 1A). This model is easier to conduct and could avoid the confounding effect from PHx on proliferation of future liver remnant (FLR).

Review#1's next question regarding that "The results section should be organized better, since phrases that could correspond to Material and Methods are presented, and others that should be included in the Discussion section."

Response: Thanks for this good question that helps us improve our paper.

Based on your comments, the results section should be organized. In brief, the first part of result introduced the establishment of appropriate PHx model. Then we stated to present the results from Figure 1 to 5. Some redundant sentences have been deleted or included in the Discussion section. In this way, the article appears to be more logical and easier to read.

Review#1's last question regarding that "Stage 2 of the ALPPS technique is normally performed 9 days after Stage 1 in humans, and some authors advise extending this period of time by a few more days. However, the authors sacrifice the rats at the 2nd and 5th postoperative day, when the hepatoblasts have not yet matured to hepatocytes. It would be advisable to make a group of rats that is sacrificed between 9-10 days."

Response: Thanks for this very helpful advice.

Previously, several animal ALPPS models have been established.[1-10] The observed period is from 4 to 7 days. Therefore, we chose the fifth day as a time point of later proliferative response. In clinical settings, the interval time between two steps are usual 1 or 2 weeks. As the species specificity, the rate of metabolism is apparently faster in rats than in humans. The observed period should be shortened correspondingly.

Definitely, such advice is very valuable which could be of great help for us to know whether the proliferation will be finished in 9-10 days. And our future work is to make a group of rats that is sacrificed between 9-10 days. Thanks for the valuable advice again.

Year	Author	Species	Time points
2014	Schlegel	Mouse	1d,2d,4d,7d
2015	Shi HW	Rat	1d,3d,7d
2015	Rocío GP	Rat	1h,1d,2d,8d,11w
2015	Kristopher PC	Porcine	7d
2013	He´ctor MAT	Rat	3d,7d,14d
2015	Wei WW	Rat	3d
2016	Magda	Mouse	12h,1d,2d,7d
2016	Michael	Mouse	1d,2d
2016	Liao MH	Rabbit	1d,3d,7d,14d
2015	Dipok KD	Rat	1d,2d,4d,7d

Review#2's first question regarding that "In the first part of the results section, the authors compare the outcome after ALPPS and extended hepatectomy. Here, the mortality rate for extended hepatectomy is at 80%, which clearly is not in line with previous publications. Indeed, it is roughly three times as high as it usually should be. Here, technical complications might be a potential bias and indeed 60% of individuals died within the first 24h, where deaths due to technical complications usually take place. Further, remaining 20% seem to be a reasonable mortality rate. The authors should reduce the respective section in the manuscript or perform the experiments again if feasible."

Response: Thanks for raising the valuable question.

We will reduce the respective section in the manuscript. Although technical complications is a rational reason for mortality, but in our study, the most suitable reason for death is the acute hepatic failure after extended hepatectomy (resection of 75%-80% of total liver). There are two reason as below. Firstly, we have done several similar rat experiments (e.g. PHx, ALPPS, bile duct ligation models). Technical complications usually occur intra-operation or early period of post-operation and the rats always do not recovered from the sedation. In our study, all rats presented autonomic activities and have apparent consciousness. Therefore, we inferred the reason for such high mortality is the acute hepatic failure although 60% rats died within 24 hours. Besides, a repeated trial was also conducted by Ying Han-Ning and Xu Ming. Only one rat could survive with such extended hepatectomy, the rest (n=10) rats were all dead within 72 hours. These experiments were performed in the initial stage of this study in July, 2015 (only 9.1% survival rate). Therefore, we adjusted the extent of liver resection and found that the medium PHx model (removal of left lateral, right and caudate lobes) presented an acceptable mortality (20%) and triggered a remarkable proliferation of FLR.

Review#2's second question regarding that "The western blot in Figure 1E (in the text falsely referred to as Figure 2E) should be quantified. Indeed, the conclusion that PCNA "appears earlier" in the ALPPS group is not 100% true. Further, the sample S1 on Day-2 seems to have a lower loading volume, as the house keeping protein is significantly lower than it is for the rest of the samples on this blot."

Response: Appreciate for this careful notice and valuable instruction that helps us modify the paper.

We feel very sorry for our mistake that the western blot in Figure 1E falsely referred to the Figure 2E. Such mistake has been corrected. Additionally, we agree with review#2's opinion that the sample S1 on Day-2 seems to have a lower loading volume.... Therefore, the statement in this section is "PCNA in the ALPPS group appeared earlier than that in PHx group". Because the house keeping protein seemed no significantly different between ALPPS and PHx group.

Review#2's next question regarding that "In the text the authors state that next to Sox9, Epcam and Lgr5 were significantly increased markers but miss to mention that they do refer to mRNA levels. Further, no statistical workup of these results is completely missing in the results section. Next, they show immunofluorescence images exclusively for Sox9. Here, the authors should include first of all a quantification and second show immunofluorescence data on the other differentially expressed markers as well."

Response: Thanks for this very helpful guidance.

According to Review#2's advice, the expression of markers of progenitor hepatic cell was mentioned with P-values in the revised manuscript. And In Figure 3, there was no significant different between sham and ALPPS group in terms of expression of Lgr5 at mRNA level. We then have corrected the statement in the revised manuscript.

For the immunofluorescence images, the Sox9 was the classical marker of hepatic progenitor cell although the Epcam was a marker of hepatoblast marker. And not all such hybrid hepatocyte were Epcam positive. Therefore, we only detected the expression of Epcam in mRNA level rather than protein level. A part of our future work is to explore the source and destiny of such progenitor cell by lineage tracing method.

Review#2's forth question regarding that "The conclusions drawn from Figure 3A are not objective at all. Again, the results should be quantified. However, while the authors report on a delayed up regulation of HNF4, they neglect the more interesting part of this figure, namely the (optically) higher abundance of PCBD1, KLF15 and CEBPA, if not also of GATA4 in ALPPS individuals at the Day-2 time point. The authors need to urgently discuss this figure, as it actually reverts their proposed theory on a lower maturity of hepatocytes after ALPPS procedure."

Response: Thanks for this valuable advice.

We are agreeing with this advice that quantified results were better. In our study, we detected the HNF, KLF15, etc at mRNA level by PCR, which suggested activity of transcription in later stage of ALPPS procedure. To further validate the delayed expression of HNF4, the IHC was performed. Taken together, the delay peak of HNF4, a classical marker of mature hepatocyte, indicated the immaturity of ALPPS-derived liver regeneration. Besides,

even the expression of hepatic transcription factor was high in early stage, this did not mean the maturity of hepatocytes after ALPPS. Theoretically, some PCBD1-dependent or KLF15-dependent functions have been mature while some HNF4-dependent functions were immature. Certainly, the higher abundance of PCBD1, KLF15, CEBPA, GATA4 might participate in programming the fate of ALPPS-induced newborn hepatocyte, which needs us to do further inquiry. Thanks for this valuable advice again, as it gives us new ideas for future research.

Review#2's fifth question regarding that "While the data on urea nitrogen are shown very nicely in Figure 4, the data on PAS, ICG and Oil Red staining are not relatable. Again, quantification and statistical workup is crucial here. However, the data on CYP-regulation seems solid and convincing to me."

Response: Thanks for this good question that helps us improve our paper.

In Figure 4, we detected the liver function to evaluate the maturity of ALPPS-derived liver regeneration. The PAS stain, OilRed stain, ICG stain, represents the capacity of glycogen synthesis and fat metabolism and ICG up-take, respectively, as well as the synthesis of urea nitrogen, the expression P450Y enzymes and albumin were an important component of mature hepatocyte function. Among, the PAS, ICG and Oil Red are not easy to perform the statistical workup. Besides, an insufficiency of urea nitrogen synthesis of ALPPS groups existed in the whole course of liver regeneration. With respect to the metabolism, the expression of CYP3A1 and CYP2B6 in ALPPS group was suppressed on the second day and the expression of CYP1A1 and CYP7B1 was down-regulated in both stages. As seen from above, these detection suggested the immaturity of newborn hepatocytes in ALPPS group.

Review#2's sixth question regarding that "When looking at the cluster analysis of gene expression, the late time point for ALPPS individuals shows a uniformly low expression of all mRNAs tested. This is rather confusing, as one would expect at least one gene involved in those central mechanisms to be up-regulated at this stage (as it is the case for Day-2 sham mice). Have the authors tested the used mRNA for a potential contamination or destruction via RNases? This might explain the confusing results. In case that the data are reliable and

that no error was found in the experiment conduction, the authors need to underline the differences between ALPPS and PHx, as this part is widely neglected for the cluster analysis."

Response: Sincerely thanks for raising the issue.

To begin with, we could guarantee that the protocol for isolating mRNA is correct. As the authors in charge of mRNA related experiments have received research training at least three years, the possibility of potential contamination or destruction via RNases is very, very low. As the review#2 said, the cluster analysis seemed to be not in line with previous results. A plausible reason, we support, is that in the initial stage of liver regeneration, almost all functional genes were up-regulated and were accompanied by an increase in the amount of hepatocyte, both in ALPPS or PHx procedure. However, in the later proliferative response, the differentiation process were activated rather than the increase in amount. In this setting, the ratio of immature hepatocyte was increased relatively in ALPPS and PHx group. For now, this is just a hypothesis to explain why the functional was high expressed in early stage but low expressed in later stage. Definitely, more efforts should be done to explore the underlying mechanism. In comparison with PHx, we found that the maturity of hepatocyte derived from ALPPS is postponed, which was in most important finding in this study. Because this could be a convincing evidence that the stage II of ALPPS should be performed prudently in patients with marginally adequate FLR, as the ALPPS-derived proliferation in volume lags behind the functional regeneration.

Review#2's last question regarding that "In the introduction, the authors refer to postoperative liver dysfunction as small-for-size syndrome (SFSS)... And In the methods section, the authors describe the isolation protocol for primary hepatocytes and report a low speed centrifugation step at 1000 rpm..."

Response: Thanks for this useful advice that helps us improve our paper.

The concept of small-for-size syndrome (SFSS) is more frequently used in the field of liver transplantation indeed. We also think that the "liver dysfunction/failure" are more appropriate in our study. Secondly, we are sorry for our mistake in terms of units. We have corrected the "rpm" into "g". Thank you for this useful advice.

Eventually, we are very grateful for editor and reviewers' efforts for reviewing our paper. These comments and instructions improve the quality of our paper efficiently. **Besides, as Billy Lu and Jun-jie Hong made a great contribution in revising this paper, could they be added into the author list?** Billy Lu, as a native speaker, was graduated from John Hopkins University and work in National Institutes of Health (NIH), American. With their help, we think the language in this study have met the criterion (Grade). If not, we are very happy to ask language editing company you recommended to polish our manuscript. **When the two authors are added, we will sign the "Copyright License Agreement".** We are very looking forward to your positive response.

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