nature portfolio

Peer Review File

FGF21 modulates immunometabolic homeostasis via the ALOX15/15-HETE axis in early liver graft injury

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the study titled “FGF21 deficiency disrupts immunometabolic homeostasis via the ALOX15/15-HETE axis to aggravate early liver graft injury”, Yang et al. describe a role for FGF21 in liver graft injury. They show that high levels of FGF21 in blood and in the graft are associated with liver graft function and survival after liver transplant, while FGF21 deficiency worsens early graft injury. They observed that absence of FGF21 is associated with ALOX15/15-HETE increased levels, triggering inflammation deleterious for the graft. On the other hand, they show that FGF21 overexpression protects from liver graft injury, notably in steatotic livers that usually tend to have lower levels of FGF21.
The study is interesting, well designed and easy to follow. However, the novelty of the results is tempered by the fact that the role of ALOX15 in liver steatosis has already been demonstrated previously (Martinez-Clemente et al. Hepatology 2010). As well as the proposition of FGF21 as a prognostic biomarker for early detection of I/R injury in patients with liver transplantation (Ye et al. Sci Rep. 2016). Here the originality remains in the link between FGF21 levels and the inhibition of ALOX15. Therefore an exploration of the molecular mechanisms inducing ALOX15 inhibition through FGF21 pathway is crucial to give more strength to the point.

Comments:

-The paper is missing a description about ALOX15 and its functions. For a better understanding of the study, a paragraph defining the role of this lipoxygenase in general and more precisely in the liver should be added in the introduction. A definition of the abbreviation of ALOX15/15-HETE is also required.
Moreover, the current literature concerning ALOX15 is absent and should be mentioned. For example, the idea that ALOX15 axis is involved in hepatic steatosis has already been described by Martinez-Clemente et al. in a Hepatology paper in 2010. This should be discussed. It is already known as well that the absence of ALOX15 improves liver inflammation in alcoholic liver disease (Queck et al. Front. Immunol 2020), which could also be commented regarding the results obtained about inflammation in this work.

-The distribution of ALOX15 being different in rodent compared to humans, this might complicate the extrapolation of ALOX15 function from mice to humans. Moreover, it also doesn’t seem clear to date whether ALOX15 in mice should be compared to the human gene ALOX15 or with ALOX12 (Ivanov Gene 2015). This should be discussed.

-How is FGF21 affecting ALOX15 levels at cellular levels? Explaining the molecular mechanisms behind would radically improve the importance and the novelty of the interactions described in this study.

-Further experiment is required to confirm that I/R injuries in FGF21 KO animals are due to ALOX15 increase. A repression of ALOX15 in FGF21 KO mice would be of interest in that sense.

Minor comments:

-Sometimes the paper lacks clarity and further explanations and abbreviations definition are required to help the reader follow the flow of the story.

-Please be accurate with genes and proteins nomenclature when referring to human or mice samples.

-It is not always clear where are the different cohorts coming from or which one is used at what time. Please clarify.

Comments figures:

Figure1:
Figure 1h shows that the levels of AST and ALT are not affected by the levels of FGF21 in non-steatotic liver transplants in human. This moderates the message of FGF21 as a novel marker for transplanted liver graft injury at least in non-steatotic patient.

Figure 4:
Here a group of WT liver transplanted in FGF21 KO mice would be interesting. Indeed, the secretion of FGF21 by other peripheral tissues like brown adipose tissue can be non-negligent. This would help confirm that the FGF21 measured in the serum comes from the transplanted liver and not from another organ crosstalk.

Figure 5:
Observing the effect of rmFGF21 administration after I/R would be interesting as the levels of FGF21 in humans were compared after transplantation versus before.

Figure 6 and supp 7:
Evidence that HFD induced hepatic steatosis in this model is not convincing. The ORO staining does not show strong lipid accumulation in the sham group under HFD. H&E sections of livers would be nice to apprehend lipid accumulation.
Explain why looking at CD11b+ and MPO+.

In fig6a I observe more steatosis in the “FGF21 high” group. Isn’t it unexpected?

Figure 7:
Why transfection so early in the process? The condition with shALOX15 reflects more the role of ALOX15 in steatosis formation than its role in I/R of already steatotic livers.
Evidence that HFD induced hepatic steatosis in this model should be shown.

Figure 8:
Why doing different timings between transgenic overexpression and recombinant protein?

Marion Peyrou

Reviewer #2 (Remarks to the Author):

This is a interesting study to examine the role of FGF21 in liver I/R injury. Although the protective role of FGF21 in several liver diseases have been well defined in previous studies, its effect in early liver graft injury is largely unclear. By analyzing appropriate human clinical

samples and mouse models, the authors identified that the elevation of FGF21 is protective against hepatic ischemic injury, which possibly dependent on the modulation of the ALOX15/15-HETE axis. The use of RNA-seq, transgenic mouse models, and AAV transfection were well done. Overall, the study revealed a novel role of FGF21 in liver graft injury. However, I still have some concerns about the study design which need to be addressed to support the entire hypothesis.

Major Concerns
1. As an autocrine factor in the living organism, the source of FGF21 upregulation in mouse I/R models should be well defined.
2. Similarly, FGF21 is an autocrine factor and its effects rely on the activation of receptors, does the protective role of FGF21 in liver I/R injury is dependent on FGFR4 in hepatocytes in the authors' models?
3. CyTOF is a powerful tool to identify the innate immune responses in mouse liver transplantation, however, the exact N value of mice in each group should be clearly pointed out in the methods part or figure legends of the article. If the N value is less that 3 in each group, other methods such as flowcytometry or IHC staining should be performed to further verify these results.
4. In the statistical analysis part of the article, the authors simply wrote "All experiments were repeated a minimum of three times", this is not a scientific writing of research papers, will the RNA-seq or CyTOF be repeated three times, separately? I suggest a detailed revision of this part of the article.
5. The liver I/R injury consists of the early acute inflammatory responses and the later regenerative responses (normally 24 hours after reperfusion), interestingly, FGF21 also shows potential to promote cell proliferation in several organs. Therefore, does FGF21 affect hepatocyte proliferation in the author' mouse models?

Minor Concers

Since FGF21 overexpression approaches were used in the study, I suggest the title of the article change to "FGF21 modulates immunometabolic homeostasis via the ALOX15/15-HETE axis to aggravate early liver graft injury" or other appropriate writings.

Reviewer #3 (Remarks to the Author):

The authors have assessed the importance of FGF21 in ischemia reperfusion of the liver using a clinical model and experimental murine models. The authors suggest a role of FGF21 in hepatic ischemia reperfusion in particular steatotic livers.

1) Abstract: I found the abstract extremely confusing. Please reorganize including a brief description of the models used.
Clinical model:
2) It is unclear what definition of EAD was used. It should be clearly stated in the methods. Is the Olthoff et al used?
3) It is unclear how you divided patients in two groups, elevated and non-elevated FGF21 serum level
4) A correlation between FGF21 serum level and graft survival is suggested, the authors

should be cautious in making such correlation, causes of graft failure should be reported.
5) Overall the clinical correlation between FGF21 serum level and clinical outcome is quite weak. Would be important to see how FGF21 correlate with other markers of graft function and injury. Is FGF21 just another maker of the severity of I/R injury? Have you assessed other biomelocular markers?
6) Regarding the clinical study (results 2.6), I could not find a definition of steatosis, the authors should define how steatosis was defined and assessed.
7) In general further details regarding the transplant surgery should be included. A standard technique and reperfusion method was used in all recipients?
8) IS very unclear when the biopsy was taken in steatotic graft, I assume that was taken not more than 30 min after reperfusion? Also is interesting that staining was positive for FGF21 shortly after reperfusion. A comment is necessary
9) Discussion: The authors briefly discussed potential clinical application of FGF21 in steatotic livers. The authors should briefly discuss potential advantage of FGF21 compared to other interventions example defatting during machine perfusion.

We would like to express our sincere gratitude to the editors and reviewers for their constructive and helpful comments to our paper. We have taken the comments on board to improve and clarify the manuscript. The detailed point-by-point responses to all comments (reviewers' comments in black, our replies in blue) are described below.

REVIEWER COMMENTS

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Comments:
1. The paper is missing a description about ALOX15 and its functions. For a better understanding of the study, a paragraph defining the role of this lipoxygenase in general and more precisely in the liver should be added in the introduction. A definition of the abbreviation of ALOX15/15-HETE is also required.

Response: Thank you for your valuable comment on our manuscript. Lipoxygenases (LOXs) are non-heme iron enzymes that catalyze the peroxidation of polyunsaturated fatty acids (PUFAs), producing hydroperoxy derivatives. They act as pro- and anti-inflammatory mediators and are involved in membrane restructuring, lipoprotein interaction, and cellular redox regulation [1,2]. *ALOX15*, encoding 15-LOX (also known as 12-LOX or 12/15-LOX), utilizes natural substrates like arachidonic acid (AA), docosahexaenoic acid (DHA), *et al*. These substrates are present either in their free state or integrated into molecules like phospholipids, glycerides, or cholesterol esters [3]. AA, a prominent ω-6 PUFA representing a

major component of the cell membrane phospholipids and the metabolic precursor of eicosanoids, is converted by *ALOX15* into 15-hydroxyeicosatetraenoic acid (15-HETE) [3]. These compounds are then metabolized into diverse bioactive molecules, contributing to the pathogenesis of several diseases [3,4].

ALOX15 has been shown to be linked with nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). Lazic *et al.* found that *Alox15* deficiency, which results in significantly lower plasma concentrations of 15-HETE, protects against chronic high-fat diet-induced steatohepatitis [5]. *Alox15* deficiency was also found to alleviate hepatic steatosis, insulin resistance, and inflammatory injury in hyperlipidemia-prone apolipoprotein E-deficient mice [6]. Moreover, Zhang *et al.* found that in alcoholic liver disease, *Alox15* knockout ameliorates alcohol-induced reactive oxygen species (ROS) production, endoplasmic reticulum (ER) stress, apoptosis, and liver injury [7]. However, the role of ALOX15 in the pathogenesis of ischemic liver disease remains unknown.

According to your suggestion, a paragraph defining the role of ALOX15 in general and more precisely in the liver has been added in the introduction (Introduction section, page 5, lines 27 to 36 and page 6, lines 1 to 10). Also, we've added the definition of the abbreviation of ALOX15/15-HETE in abstract and main text. ALOX15/15-HETE is described as follow: the enzyme arachidonate 15 lipoxygenase (ALOX15)/15-hydroxy eicosatetraenoic acid (15-HETE).

2. Moreover, the current literature concerning ALOX15 is absent and should be mentioned. For example, the idea that ALOX15 axis is involved in hepatic steatosis has already been described by Martinez-Clemente et al. in a Hepatology paper in 2010. This should be discussed. It is already known as well that the absence of ALOX15 improves liver inflammation in alcoholic liver disease (Queck et al. Front. Immunol 2020), which could also be commented regarding the results obtained about inflammation in this work.

Response: Thank you very much for your previous comments that helped us improve this manuscript. ALOX15 and its metabolites have a significant impact in the fate of hepatic pathologies. Queck *et al.* showed that the lack of *Alox15* worsened parameters of liver disease and increased hepatic neutrophil and $CD4^+$ regulatory T-cells infiltration in alcoholic hepatitis, while Lipoxin A4 injection attenuated these parameters of disease progression in *Alox15*$^{-/-}$ mice [8]. However, Zhang *et al.* found that activation of ALOX15/13-hydroxyoctadecadienoic acid (13-HODE) circuit critically mediates the pathogenesis of alcoholic liver disease (ALD), *Alox15* knockout ameliorates alcohol-induced ROS production, ER stress, apoptosis, and liver injury [7]. Their data suggested that ALOX15 is a potential molecular target for treatment of ALD. Different from the mechanism in ALD, our present study was able to define that ALOX15 was an important mediator of graft injury and noticed that 15-HETE, which is involved in the AA pathway, was one of the most upregulated

metabolites in *Fgf21* KO livers after I/R treatments. Consistent with Wang *et al.*'s data showing that an ALOX15 inhibitor reduced macrophage recruitment and inflammation in mice [9], we also observed decreased intrahepatic macrophage and neutrophil infiltration and alleviated liver injury following ALOX15 inhibition in I/R-insulted liver. These results suggested that the ALOX15/15-HETE axis is a key pathway regulating graft injury in LT.

In addition to the protective effects against hepatic inflammation and cell injury, Martinez-Clemente *et al.*'s findings also showed a decrease in hepatic steatosis in *ApoE*$^{-/-}$ mice lacking *Alox15* [6]. The observed antisteatotic effect is more likely due to a combination of actions on pivotal pathways that drive the progression to hepatic steatosis, notably those associated with insulin resistance. Therefore, to avoid the condition with shAlox15 reflecting more the role of *Alox15* in steatosis formation, we minimized the transfection time to three weeks before I/R injury in steatotic livers. As shown in our study, no significant change in steatosis and lipid accumulation was found in HFD-fed mice between with and without shAlox15 either in sham or I/R groups (Supplementary Fig. 9a-c).

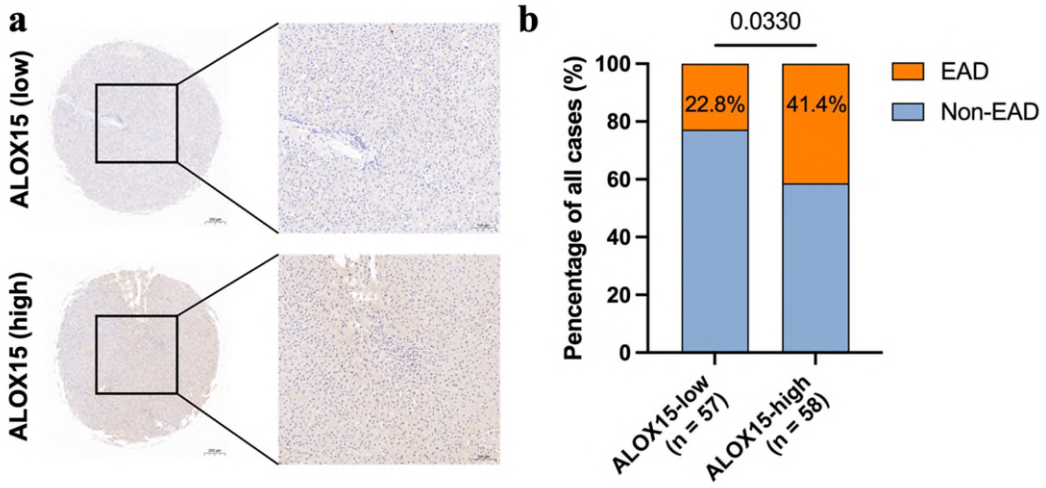
The current literatures concerning ALOX15 have been discussed in the revised manuscript (Discussion section, page 12, lines 36 and page 13, lines 1 to 30). Thank you for your constructive comments.

3. The distribution of ALOX15 being different in rodent compared to humans, this might complicate the extrapolation of ALOX15 function from mice to humans. Moreover, it also doesn't seem clear to date whether ALOX15 in mice should be compared to the human gene ALOX15 or with ALOX12 (Ivanov Gene 2015). This should be discussed.

Response: We appreciate your constructive comment. The human reticulocyte type 12/15-LOX (encoded by *ALOX15* gene) produces predominantly 15(S)-HETE and only little amounts of 12(S)-HETE (ratio of 9:1), while its murine orthologue leukocyte-type 12-LOX (encoded by *Alox15* gene), produces small amounts of 15(S)-HETE and primarily 12(S)-HETE (ratio of 1:3) [3]. However, both these enzymes share 73% amino acid similarity, a similar expression pattern and largely overlap in their known biological effects [10-12]. Moreover, the ALOX15 orthologs are somewhat unique since they are capable of oxygenating complex ester lipids (lipoproteins, biomembranes). There is hardly any functional similarity between mouse *Alox15* and human *ALOX12* in this case [13-15]. A detailed analysis of the structure and organization of the lipoxygenase genes in the human and mouse also suggest that the mouse leukocyte-type 12-LOX and the human reticulocyte-type 12/15-LOX are orthologous enzymes [16,17]. Usually, enzyme orthologs fulfill similar functions across various organisms and thus mouse *Alox15* may serve as the functional equivalent of human *ALOX15* [11].

To further validate our exploration from mice to humans, ALOX15 protein levels were also determined using IHC in pre-transplant liver graft biopsies from Cohort 2. According to

the average integrated optical density (IOD) of IHC staining, the 115 cases were divided into a low ALOX15 group and a high ALOX15 group (Supplementary Fig. 3a). The high ALOX15 grafts had a significantly higher incidence of EAD than the low ALOX15 grafts (41.4% vs. 22.8%, $p = 0.0330$) (Supplementary Fig. 3b). This result indicated that ALOX15 has potential clinical significance in early liver graft injury. Thank you once again for your valuable suggestions, we have added these contents in the manuscript (Results section, page 8, lines 2 to 7 and Discussion section, page 13, lines 15 to 23).



Supplementary Fig. S3

4. How is FGF21 affecting ALOX15 levels at cellular levels? Explaining the molecular mechanisms behind would radically improve the importance and the novelty of the interactions described in this study.

Response: Thank you very much for your previous comments that helped us improve this manuscript. To evaluate the putative mechanisms in a cell-type specific manner, we assessed the modulatory function of rmFGF21 in H/R-stressed murine hepatocyte cultures. We observed increased FGF21 expression and apoptosis in AML12 after H/R treatment (Fig. 5e). The phosphatidylinositol-3-kinase (PI3K)/AKT and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathways are well known to be regulated by FGF21, which are involved in regulating various metabolic processes [18,19]. To determine whether the ERK1/2 pathway is downstream of rmFGF21, we first analyzed the phosphorylation levels of ERK1/2 and found that rmFGF21 treatment increased the phosphorylation. Meanwhile, rmFGF21 also reduced the levels of ALOX15 while activating the ERK1/2 pathway, and such effects were diminished by treatment with an ERK1/2 specific inhibitor SCH772984 (Fig. 5f). By contrast, inhibition by a PI3K specific inhibitor LY294002 had no such effects (Supplementary Fig. 7c).

FGF receptors (FGFRs), mainly FGFR4, which is expressed in liver, and FGFR1, which is predominantly expressed in adipose tissues, are considered the primary mediators of the metabolic effects of FGF21 and FGF19 [20]. To distinguish which FGFR(s) mediate the activation of the ERK 1/2 pathway and the inhibition of ALOX15 induced by rmFGF21, we used selective FGFR1 tyrosine kinase inhibitor PD166866. We found that rmFGF21 retained the inhibitory effect on apoptosis under these conditions (Supplementary Fig. 7d), indicating that hepatic FGFR1 likely does not mediate the protective activity of rmFGF21. By contrast, the phosphorylation of FGFR4 and the inhibition of ALOX15 induced by rmFGF21 were abolished by treatment with the FGFR4-specific inhibitor FGF401 (Fig. 5g). These data indicated that hepatic FGFR4, but not FGFR1, mediates the protective effect of rFGF21.

Taken together, pharmacological administration of rmFGF21 can significantly ameliorate hepatic I/R injury. Mechanistically, FGF21 activates the FGFR4-ERK1/2 pathway, modulating ALOX15 levels to achieve these beneficial effects on hepatocytes.

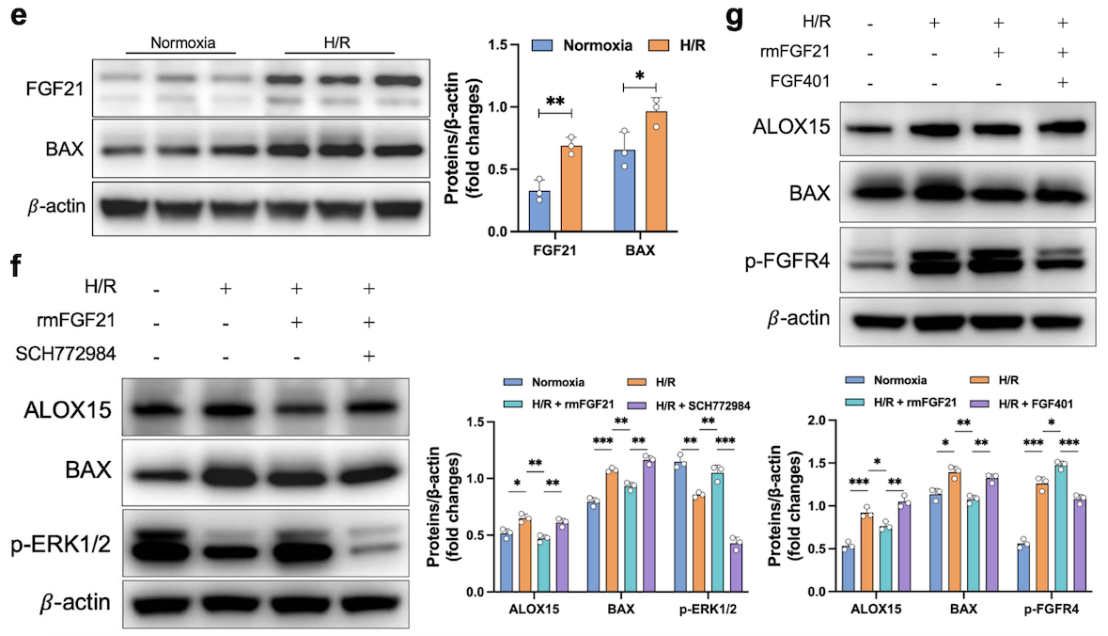
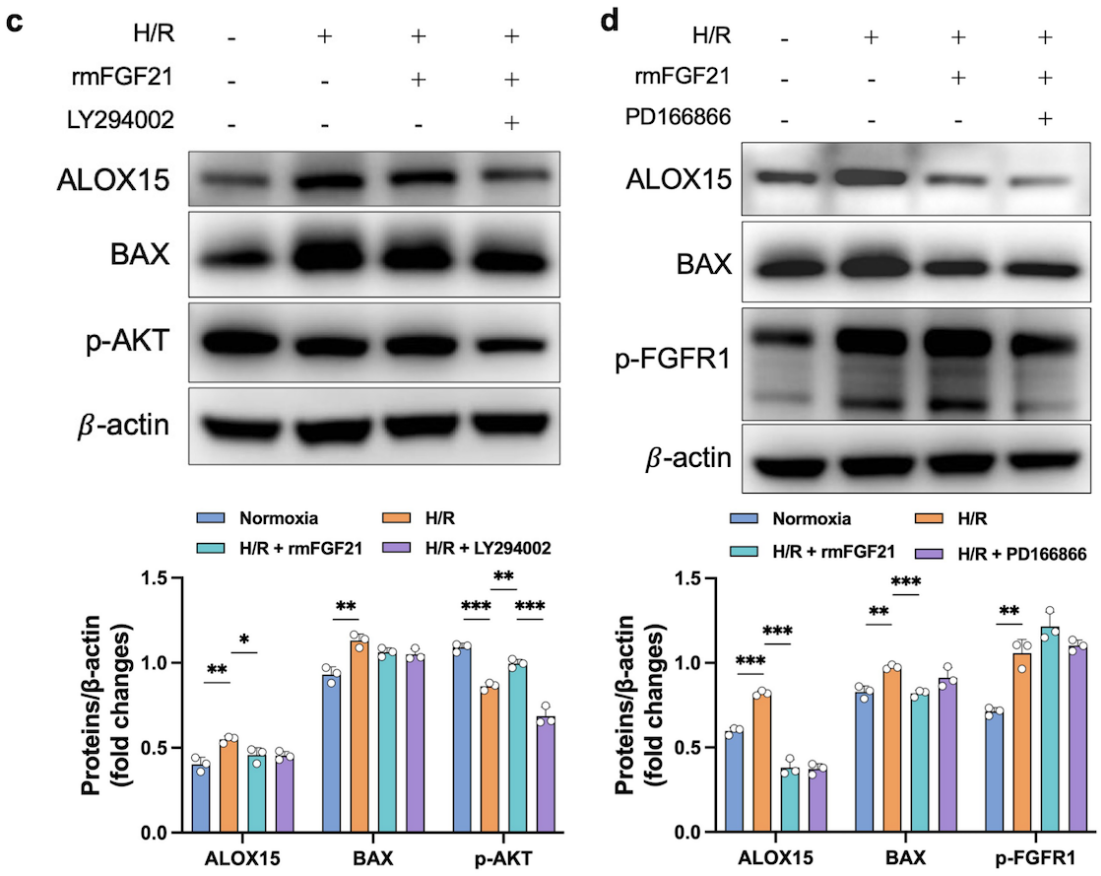


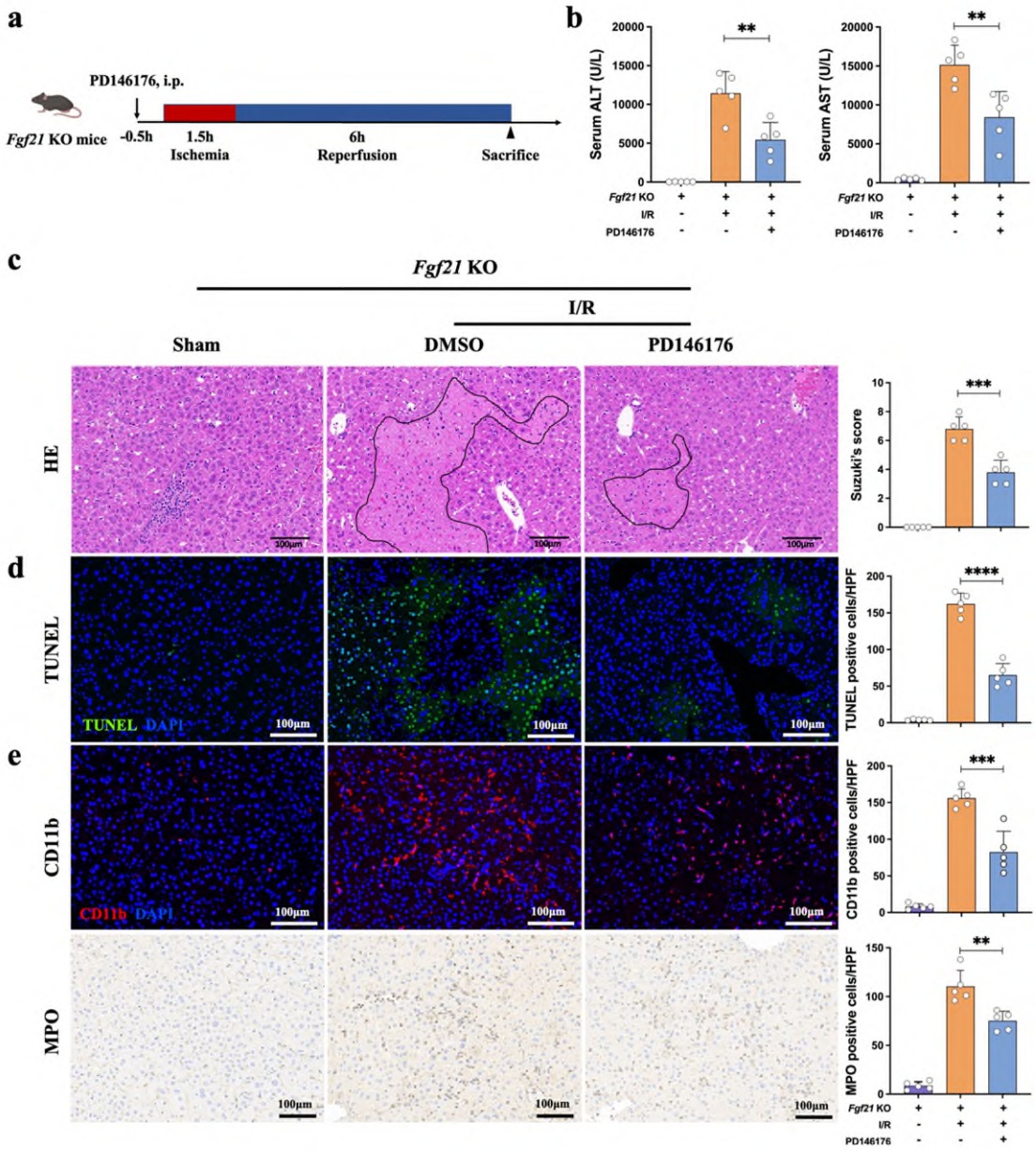
Fig. 5e-g



Supplementary Fig. 7c-d

5. Further experiment is required to confirm that I/R injuries in FGF21 KO animals are due to ALOX15 increase. A repression of ALOX15 in FGF21 KO mice would be of interest in that sense.

Response: We really appreciate this comment and recognize that additional animal experiments to support our findings will be needed. To further confirm that I/R injuries in *Fgf21* KO mice are due to ALOX15 increase, ALOX15 inhibitor was used in *Fgf21* KO mice (Supplementary Fig. 4a). *Fgf21* KO mice treated with PD146176 also showed decreased ALT/AST levels and less necrosis on H&E staining (Supplementary Fig. 3b-c). Reduced apoptosis and inflammatory cell infiltration were also observed in the PD146176-treated *Fgf21* KO livers (Supplementary Fig. 4d-e). We've revised the manuscript accordingly in the results part (Results section, page 8, lines 16 to 21). Thanks again for your suggestions, we hope these modifications will enhance the quality of our manuscript.



Supplementary Fig. S4

Minor comments:

-Sometimes the paper lacks clarity and further explanations and abbreviations definition are required to help the reader follow the flow of the story.

Response: Thank you for your comprehensive feedback. We've made the following enhancements to ensure clarity and coherence in the paper:

(1) Provide the abbreviations for certain terms, such as ALOX15/15-HETE, CIT, DWIT and *et al.*

(2) Specify the exact N value of mice of each group in CyTOF analysis.

(3) Clarify the timing of the biopsy in steatotic grafts.
(4) Incorporate additional information regarding the cohorts involved and elucidate on the transplant surgery procedures.
These revisions will be made to ensure a clearer and more comprehensive presentation of our research findings.

-Please be accurate with genes and proteins nomenclature when referring to human or mice samples.

Response: We really appreciate your correction. According to your advice, we have adjusted the names of genes and proteins described in our manuscript as following rules: (1) Human genes are typically named using italicized uppercase letters and numbers, such as *FGF21* or *ALOX15*. (2) Mice genes are often named using italicized letters and capitalized first letter, such as *Fgf21* or *Alox15*. (3) Human and mice proteins are named using italicized uppercase letters and numbers derived from the gene name (e.g., FGF21, ALOX15 protein). Thank you.

-It is not always clear where are the different cohorts coming from or which one is used at what time. Please clarify.

Response: Thank you for your valuable suggestions. We have emphasized the description in methods part, results part and figure legends. All the included recipients from January 2015 to December 2018 received standard procedure of LT in the First Affiliated Hospital, Zhejiang University School of Medicine. To detect the dynamic changes in peripheral FGF21 before and 2 h after reperfusion, serum samples were randomly collected from 88 cases (Cohort 1). Pre-transplant biopsies were obtained from the left liver lobe after liver cold storage at back table (before implantation) from a corresponding 193 cases. Of these, 115 cases (Cohort 2) were enrolled to analyze the general association between FGF21 and clinical outcomes. Another independent cohort of 78 cases (Cohort 3) was exclusively steatotic and was used to analyze the impact of FGF21 on steatotic grafts. Baseline characteristics and clinicopathological information of Cohort 1-3 are shown in Supplementary Table 2-4, respectively.

Comments figures:

Figure 1:
Figure 1h shows that the levels of AST and ALT are not affected by the levels of FGF21 in non-steatotic liver transplants in human. This moderates the message of FGF21 as a novel marker for transplanted liver graft injury at least in non-steatotic patient.

Response: Thank you for your constructive comment. As mentioned in our study, we utilized pre-transplant donor liver samples to investigate the relationship between pre-transplant FGF21 level and short-term outcomes. However, our analysis of a limited sample size (n = 55) revealed that the levels of AST and ALT after transplantation are not affected by the levels of FGF21 in pre-transplant non-steatotic donor livers. This finding suggests that factors other than pre-transplant FGF21 expression may contribute more prominently to post-transplant AST and ALT levels in this specific context. Considering all the present clinical correlation analyses, we acknowledge that assessing post-transplant FGF21 levels or dynamic change between pre- and post-transplant FGF21 expression would provide a more comprehensive understanding of FGF21 as a potential novel marker for transplanted liver graft injury. We've revised the manuscript accordingly to reflect these considerations and provide a clearer interpretation of our findings in the discussion part (Discussion section, page 12, lines 22 to 29). Thank you once again for your insightful feedback.

Figure 4:
Here a group of WT liver transplanted in FGF21 KO mice would be interesting. Indeed, the secretion of FGF21 by other peripheral tissues like brown adipose tissue can be non-negligent. This would help confirm that the FGF21 measured in the serum comes from the transplanted liver and not from another organ crosstalk.

Response: We appreciate this comment and find it scientifically intriguing. Therefore, we added an OLT group of WT >> *Fgf21* KO mice and a sham group of *Fgf21* KO mice in this experiment (Fig. 4a). Serum ALT and AST levels were significantly elevated in WT mice that received *Fgf21* KO liver grafts, while no elevation was observed in *Fgf21* KO mice that received WT liver grafts (Fig. 4b). As expected, serum FGF21 in the sham group of *Fgf21* KO mice was completely depleted. Meanwhile, a significantly decreased serum FGF21 level was found in WT mice that received *Fgf21* KO liver grafts compared to *Fgf21* KO mice that received WT liver grafts (Fig. 4c), indicating the FGF21 measured in the serum primarily originates from the transplanted liver. Thank you for your valuable suggestions.

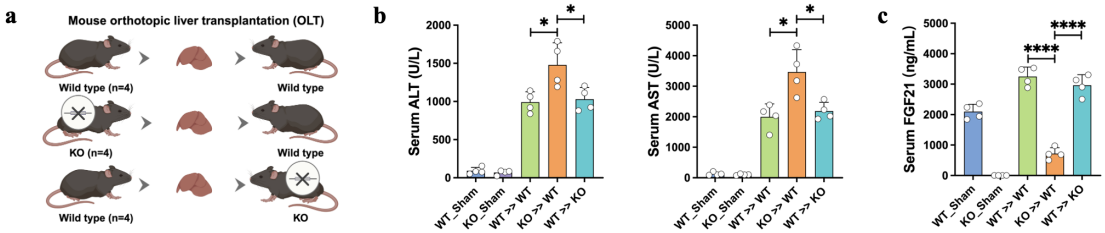


Fig. 4a-c

Figure 5:
Observing the effect of rmFGF21 administration after I/R would be interesting as the levels of FGF21 in humans were compared after transplantation versus before.

Response: We appreciate your constructive comment. According to your suggestions, we've measured the serum FGF21 level of these groups. As shown in Fig. 5c, I/R groups displayed a significant increase in serum FGF21 levels compared to the sham group. Moreover, the rmFGF21 group demonstrated the highest serum FGF21 levels after I/R. These results suggested that I/R insult can induce the expression of FGF21, and rmFGF21 treatment can further elevate serum FGF21 levels in mice subjected to I/R. Consistent with our clinical findings, the dramatic elevation of FGF21 could exert a protective adaptive response. Thank you.

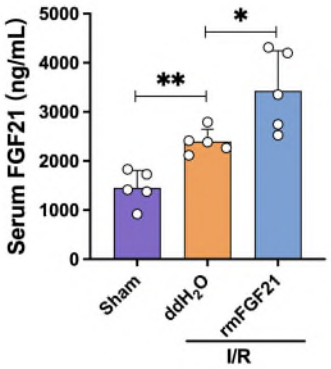
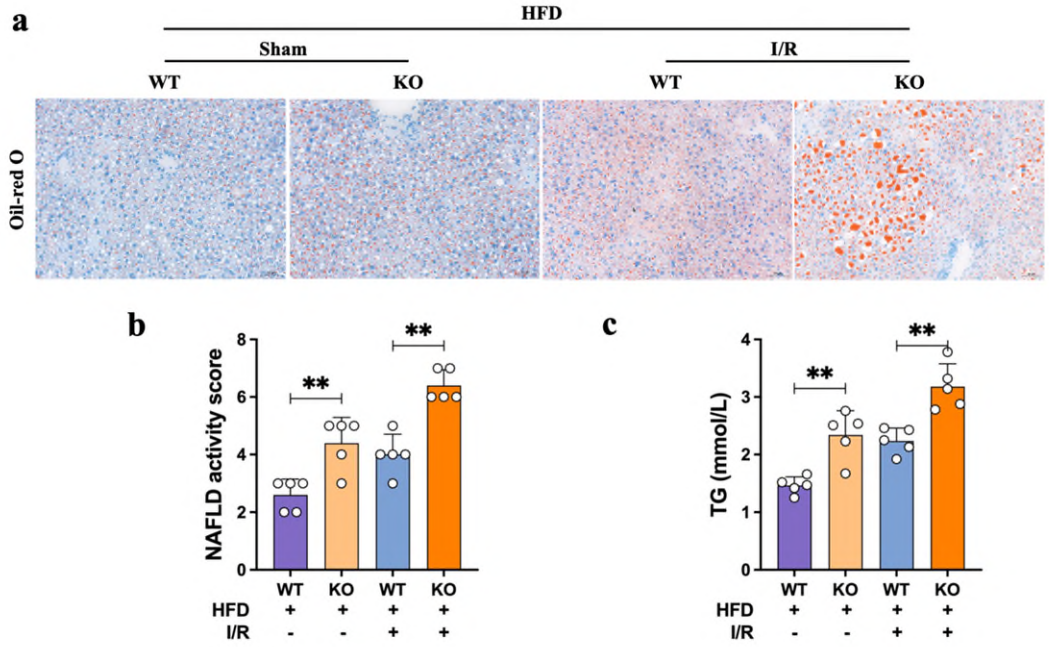


Fig. 5c

Figure 6 and supp 7:
Evidence that HFD induced hepatic steatosis in this model is not convincing. The ORO staining does not show strong lipid accumulation in the sham group under HFD. H&E sections of livers would be nice to apprehend lipid accumulation.

Response: Thank you for your valuable suggestions. We think that this variation is attributed to the individual difference within the mice group. Following your advice, we've adjusted the representative images of ORO staining in the sham group under HFD accordingly (Supplementary Fig. S8). Non-alcoholic fatty liver disease (NAFLD) activity score (NAS) was the histological tool used to assess NAFLD severity based on steatosis, lobular inflammation and hepatocellular ballooning [21]. Therefore, H&E and ORO staining were used in our study for routine histological evaluation and assessment of NAS score, as previously done for assessment of NAFLD severity [22]. The detail of NAS was also described in the

Supplementary methods section (page 3, lines 18 to 22).



Supplementary Fig. S8

Explain why looking at $CD11b^+$ and MPO^+.

Response: We really appreciate this comment and recognize that additional explanation will be needed. After reperfusion, neutrophils and macrophages are activated and accumulated in the liver, which were considered central factors leading to the liver injury [23]. CD11b, a pan-macrophage marker, was shown to mediate macrophage adhesion, migration, chemotaxis, and accumulation during inflammation [24,25]. In addition, cytosolic myeloperoxidase (MPO) can catalyze the formation of hypochlorous acid/hypochlorite and oxidizing species and is considered a reliable marker of neutrophil activity [26]. Therefore, we performed IHC and IF using CD11b and MPO antibodies to evaluate the infiltration of macrophages and neutrophils.

In fig6a I observe more steatosis in the “FGF21 high” group. Isn’t it unexpected?

Response: Thank you for your previous comment. To demonstrate whether there is more steatosis in the “FGF21 high” group, we conducted a statistical analysis comparing the degree of steatosis between the “FGF21 high” and “FGF21 low” groups and found no significant difference (Fig. 6b). Based on this analysis, we have made adjustments to the figure to reflect this finding accurately. We appreciate your attention to detail and will ensure that the revised manuscript reflects these adjustments appropriately.

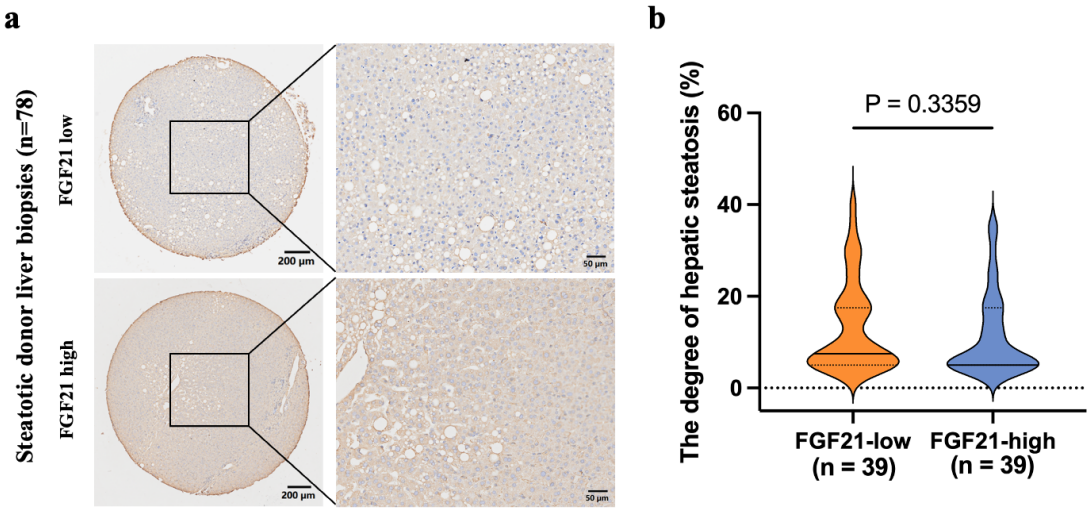


Fig. 6a-b

Figure 7:

Why transfection so early in the process? The condition with shALOX15 reflects more the role of ALOX15 in steatosis formation than its role in I/R of already steatotic livers. Evidence that HFD induced hepatic steatosis in this model should be shown.

Response: We really appreciate your valuable comments. Following your advice, we minimized the transfection time to three weeks before I/R injury in steatotic livers to avoid the condition with shAlox15 reflecting more the role of *Alox15* in steatosis formation. We observed a decrease of 15-HETE content in the *Alox15*-KD steatotic livers (Fig. 7b). The *Alox15*-KD steatotic livers showed higher resistance to I/R injury, which was demonstrated as improved liver function and alleviated histopathological liver damage (Fig. 7c-e). The *Alox15*-KD steatotic livers had reduced intrahepatic infiltration of $CD11b^+$ and MPO^+ cells (Fig. 7f). Moreover, no significant change in steatosis and lipid accumulation was found in HFD-fed mice between with and without shAlox15 either in sham or I/R groups (Supplementary Fig. 9d-e). Thanks again for your valuable suggestions, we hope these modifications will enhance the quality of our manuscript.

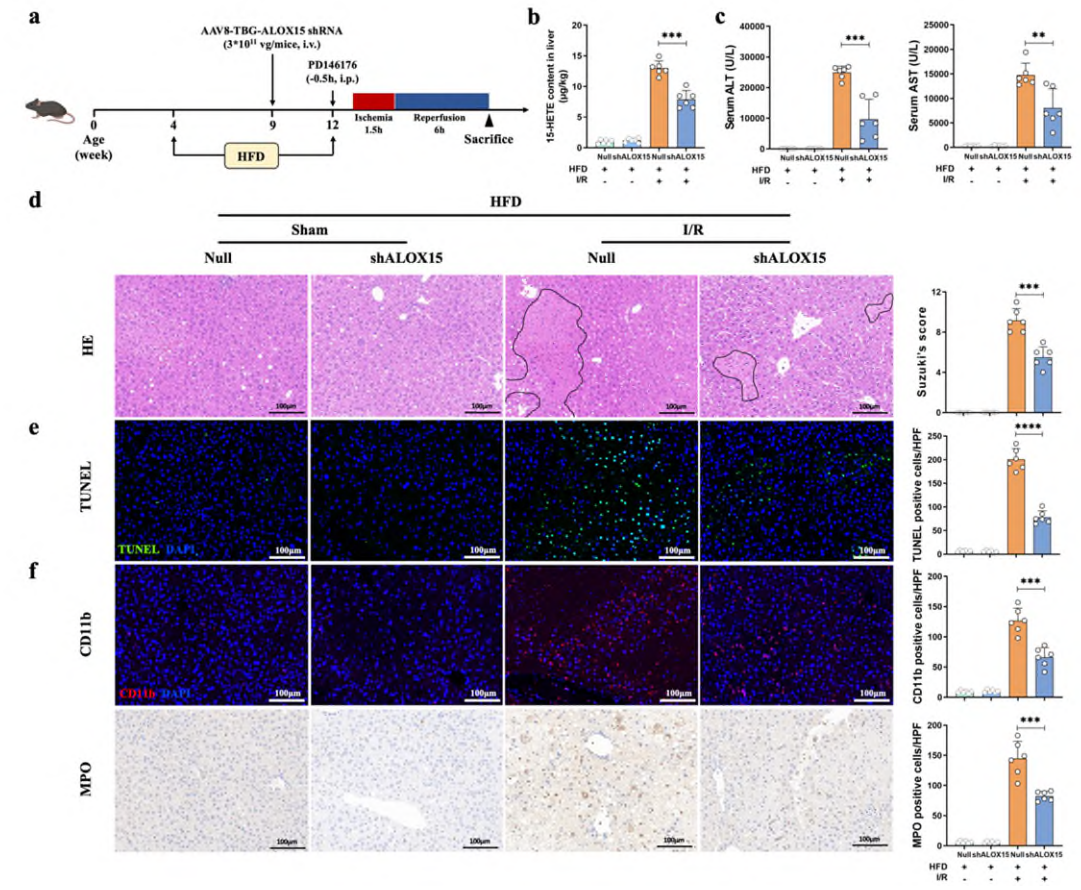
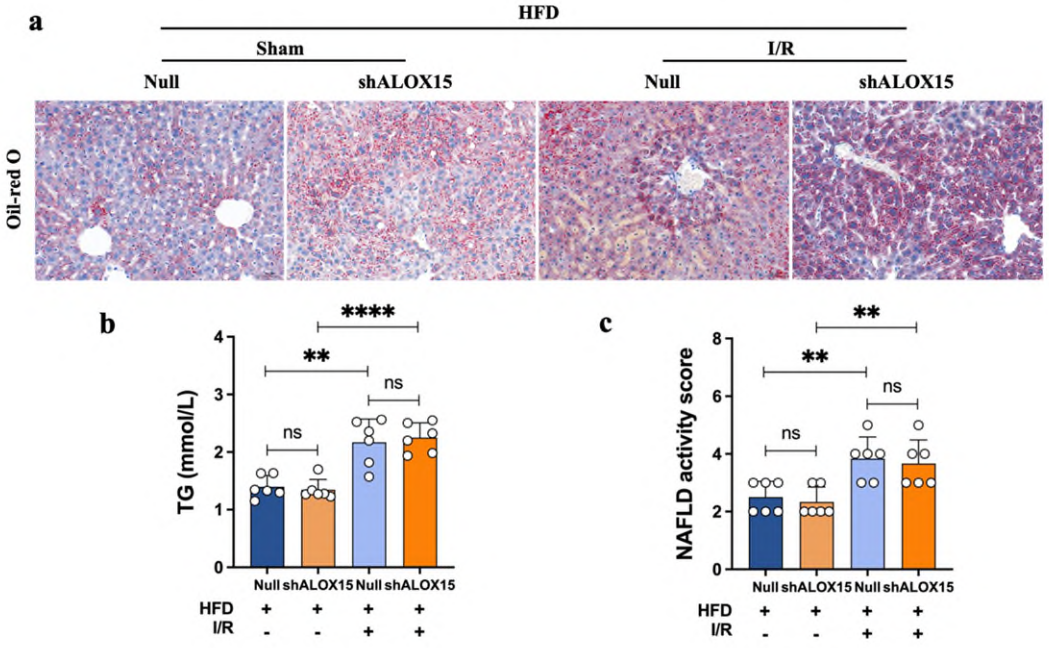


Fig. 7



Supplementary Fig. S9

Figure 8:

Why doing different timings between transgenic overexpression and recombinant protein?

Response: Thank you very much for your previous comments. Since standard AAV-mediated transgene expression peaks at approximately 3-4 weeks posttreatment and then achieves a steady state [27,28], we used 4-week-old mice for AAV injections. Then, HFD diet feeding began and was maintained for two months. Regarding the administration of recombinant protein, previous studies demonstrated that rmFGF21 at a dose of 0.5 mg/kg/d for two weeks (owing to its short half-life) effectively improved the metabolic state of mice fed with HFD [29]. These two treatments effectively reduced liver injury, apoptosis, and inflammation in steatotic livers after I/R treatment, and reversed lipid accumulation and triglyceride levels in the liver (Fig. 8 and Supplementary Fig. 10). Therefore, we adopted this dosage regimen in our present study, considering our new paradigm of killing two birds with one stone may be potentially interpreted in the preservation and repair of steatotic liver grafts.

Reviewer #2 (Remarks to the Author):

This is an interesting study to examine the role of FGF21 in liver I/R injury. Although the protective role of FGF21 in several liver diseases have been well defined in previous studies, its effect in early liver graft injury is largely unclear. By analyzing appropriate human clinical samples and mouse models, the authors identified that the elevation of FGF21 is protective against hepatic ischemic injury, which possibly dependent on the modulation of the ALOX15/15-HETE axis. The use of RNA-seq, transgenic mouse models, and AAV transfection were well done. Overall, the study revealed a novel role of FGF21 in liver graft injury. However, I still have some concerns about the study design which need to be addressed to support the entire hypothesis.

Major Concerns

1. As an autocrine factor in the living organism, the source of FGF21 upregulation in mouse I/R models should be well defined.

Response: We appreciate this comment and think scientifically it is a very interesting question. We firmly believe that the liver is the dominant source of circulating FGF21. Although FGF21 expression has also been observed in other tissues, such as muscle and adipose tissue, their contribution to circulating levels of FGF21 remains uncertain. However, previous studies reported that fasting or sympathetic activation-induced *Fgf21* mRNA in the liver and elevated FGF21 in circulation were completely abolished in liver-specific FGF21 knockout mice [30,31]. Moreover, data from tissue profiling in mice indicated that the increase in FGF21 expression due to drug-induced hepatotoxicity primarily occurred specifically in the

liver, but not in muscle, adipose tissue, heart, and other tissues, suggesting that the liver is the major contributor to the markedly elevated serum FGF21 levels [32].

To further support the notion that liver is the predominant contributor to circulating levels of FGF21 after I/R injury. We added a group of WT >> *Fgf21* KO mice and a sham group of *Fgf21* KO mice in mouse OLT experiment (Fig. 4a). Serum ALT and AST levels were significantly elevated in WT mice that received *Fgf21* KO liver grafts, while no elevation was observed in *Fgf21* KO mice that received WT liver grafts (Fig. 4b). As expected, serum FGF21 in the sham group of *Fgf21* KO mice was completely depleted. Meanwhile, a significantly decreased serum FGF21 level was found in WT mice that received *Fgf21* KO liver grafts compared to *Fgf21* KO mice that received WT liver grafts (Fig. 4c), indicating the FGF21 measured in the serum primarily originates from the transplanted liver. Thank you for your valuable suggestions.

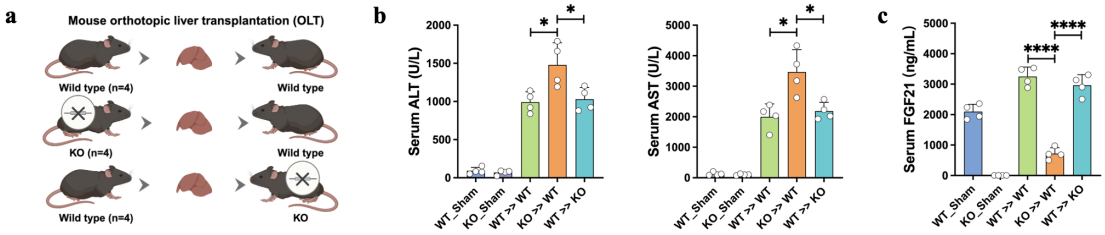


Fig. 4a-c

2. Similarly, FGF21 is an autocrine factor and its effects rely on the activation of receptors, does the protective role of FGF21 in liver I/R injury is dependent on FGFR4 in hepatocytes in the authors' models?

Response: Thank you very much for your previous comments that helped us improve this manuscript. To evaluate the putative mechanisms in a cell-type specific manner, we assessed the modulatory function of rmFGF21 in H/R-stressed murine hepatocyte cultures. We observed increased FGF21 expression and apoptosis in AML12 after H/R treatment (Fig. 5e). FGF receptors (FGFRs), mainly FGFR4, which is expressed in liver, and FGFR1, which is predominantly expressed in adipose tissues, are considered the primary mediators of the metabolic effects of FGF21 and FGF19 [20]. To distinguish which FGFR(s) mediate the activation of the ERK 1/2 pathway and the inhibition of ALOX15 induced by rmFGF21, we used selective FGFR1 tyrosine kinase inhibitor PD166866. We found that rmFGF21 retained the inhibitory effect on apoptosis under these conditions (Supplementary Fig. 7d), indicating that hepatic FGFR1 likely does not mediate the protective activity of rmFGF21. By contrast, the phosphorylation of FGFR4 and the inhibition of ALOX15 induced by rmFGF21 were abolished by treatment with the FGFR4-specific inhibitor FGF401 (Fig. 5g). These data indicated that hepatic FGFR4, but not FGFR1, mediates the protective effect of rmFGF21.

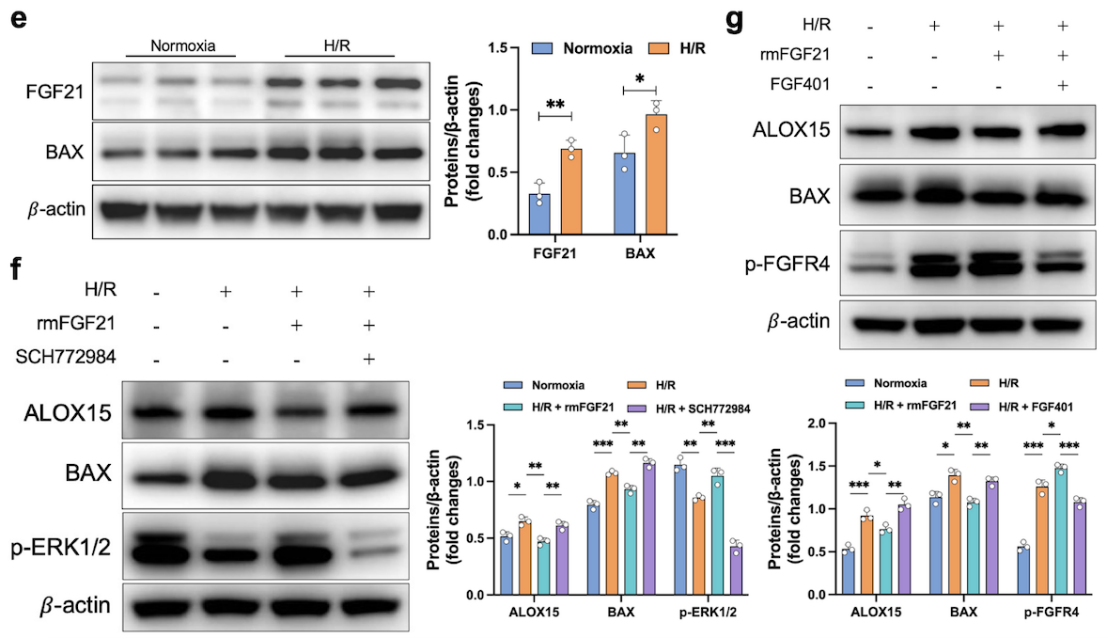
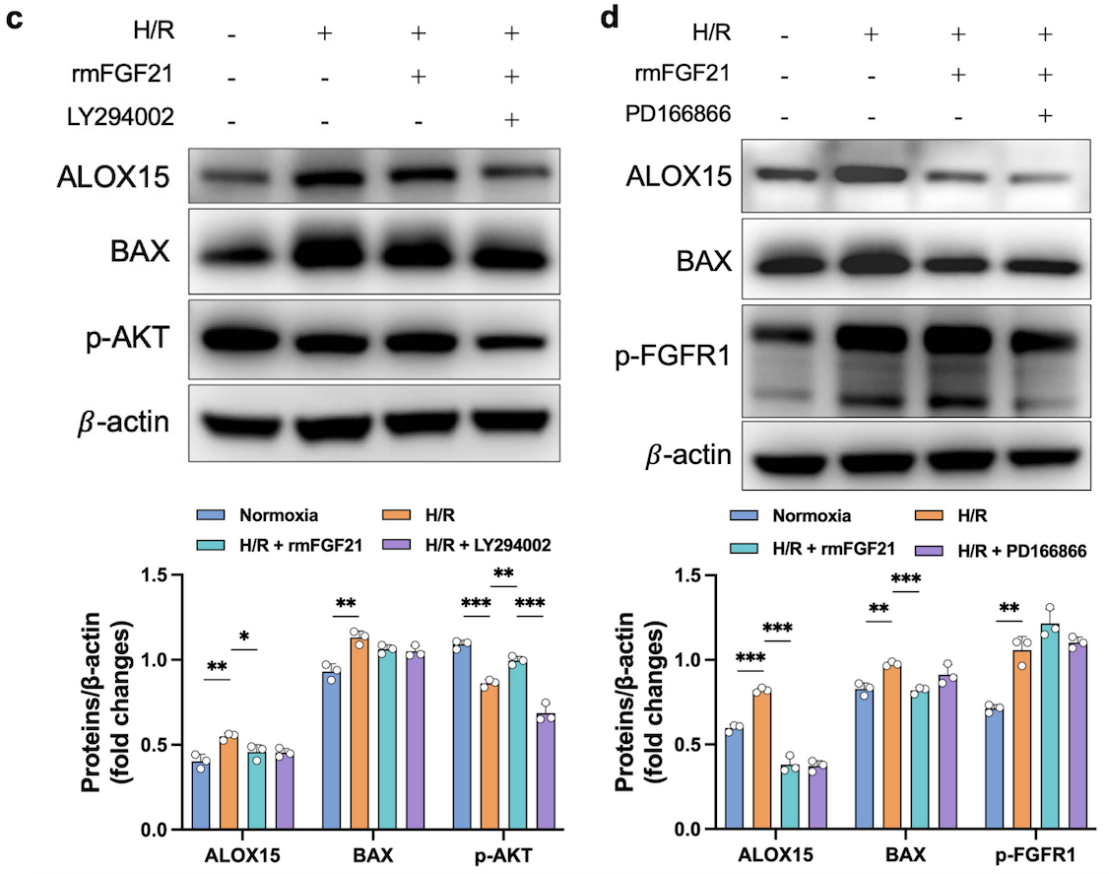


Fig. 5e-g



Supplementary Fig. 7c-d

3. CyTOF is a powerful tool to identify the innate immune responses in mouse liver transplantation, however, the exact N value of mice in each group should be clearly pointed

out in the methods part or figure legends of the article. If the N value is less that 3 in each group, other methods such as flowcytometry or IHC staining should be performed to further verify these results.

Response: Thank you for your valuable suggestions. In our study, the CyTOF data of livers isolated from WT >> WT and KO >> WT (n = 4/group) were used to analysis the change of innate immune responses. According to your suggestions, we have clearly pointed out the exact N value of each group in the methods part and figure legends (Materials and Methods section, page 17, lines 31).

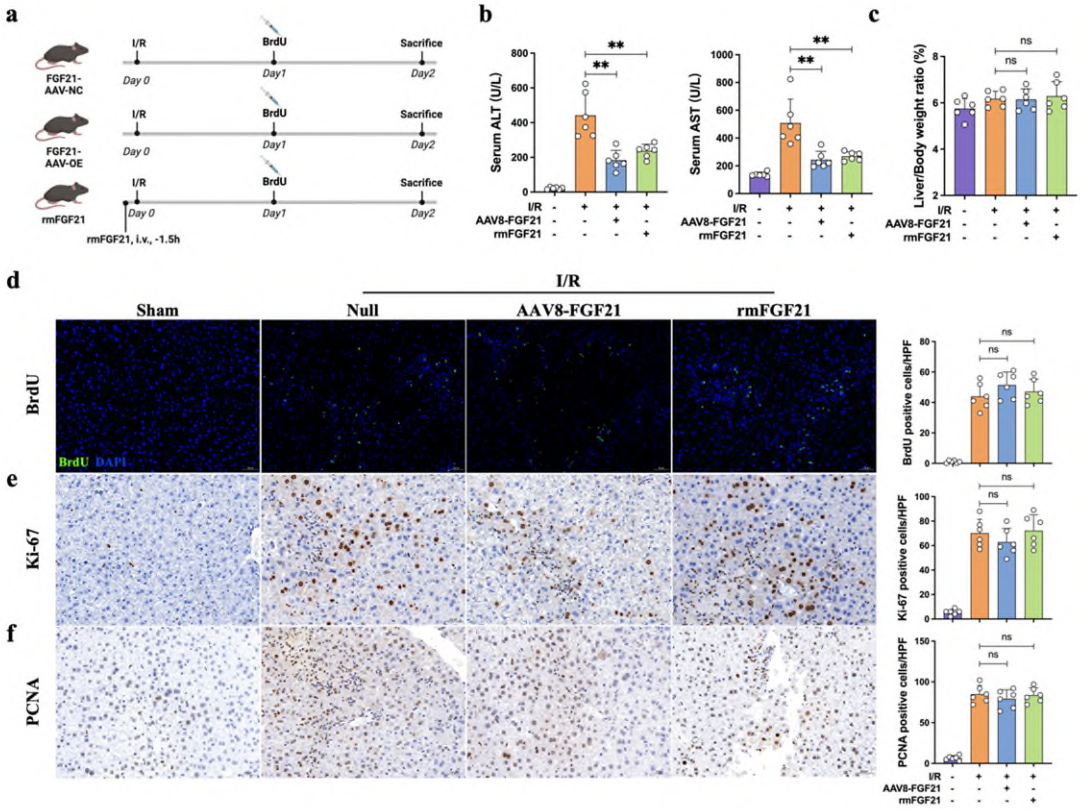
4. In the statistical analysis part of the article, the authors simply wrote “All experiments were repeated a minimum of three times”, this is not a scientific writing of research papers, will the RNA-seq or CyTOF be repeated three times, separately? I suggest a detailed revision of this part of the article.

Response: Thanks for your valuable correction. In our study, both *in vitro* and *in vivo* experiments were conducted with at least three biological replicates per condition, except for RNA-seq, metabolomics and CyTOF, which were performed once. However, at least four biologically independent samples per group were measured in the omics profiling. A revision of this part has been made in our manuscript (Materials and Methods section, page 18, lines 27 to 30).

5. The liver I/R injury consists of the early acute inflammatory responses and the later regenerative responses (normally 24 hours after reperfusion), interestingly, FGF21 also shows potential to promote cell proliferation in several organs. Therefore, does FGF21 affect hepatocyte proliferation in the author’ mouse models?

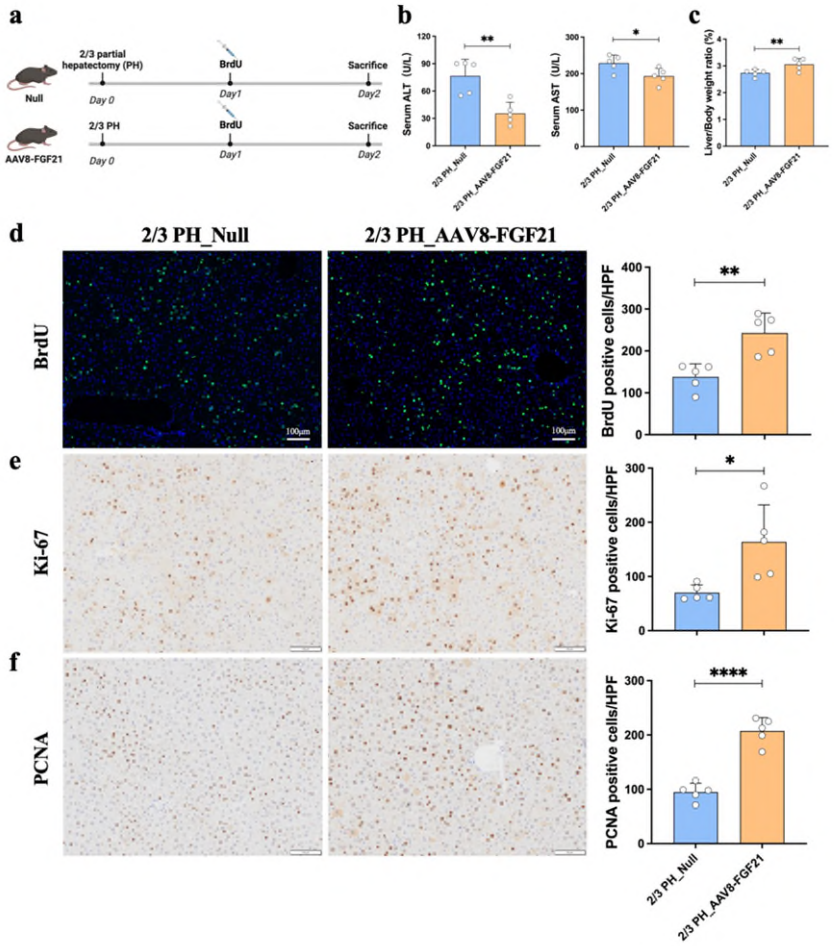
Response: We fully agree with your point regarding the importance of regenerative responses after I/R injury. To further investigate the impact of FGF21 on regeneration, we have designed a series of *in vivo* experiments.
(1) To study the impact of FGF21 on hepatocyte proliferation after I/R injury, we developed two other types of mouse models using transfection with AAV8-hAAT-*Fgf21* (liver-specific overexpression) or administration of rmFGF21 before the I/R procedure in mice (Response letter Fig. 1a). Compared to the control group, the *Fgf21*-overexpressing livers or rmFGF21-treated livers had decreased ALT and AST in serum two days after I/R (Response letter Fig. 1b). However, no significant change in liver/body weight ratio was found among I/R groups (Response letter Fig. 1c). Moreover, we evaluated hepatocyte proliferation by immunohistochemically labeling proliferation markers, such as bromodeoxyuridine (BrdU), Ki-67, and proliferating cell nuclear antigen (PCNA). From the comparative analysis of

BrdU, Ki-67, and PCNA with the control group, transgenic overexpression of hepatic *Fgf21* or administration of rmFGF21 couldn't achieve the significant features of liver regeneration after I/R (Response letter Fig. 1d-f).



Response letter Fig. 1

(2) The 2/3 partial hepatectomy (PH) is a clinically relevant approach to induce liver regeneration. Therefore, to better study the impact of FGF21 on hepatocyte proliferation, we developed a mouse model using transfection with AAV8-hAAT-*Fgf21* before the 2/3 PH procedure (Response letter Fig. 2a). Compared to the control group, the transgenic overexpression of hepatic *Fgf21* group had a lower ALT and AST level in serum and a higher liver/body weight ratio (Response letter Fig. 2b-c). Furthermore, the comparative analysis of BrdU, Ki-67, and PCNA also demonstrated that the transgenic overexpression of hepatic *Fgf21* significantly enhanced liver regeneration after a 2/3 PH (Response letter Fig. 2d-f).



Response letter Fig. 2

The different effects of FGF21 on regeneration between these two models may be attributed to the distinct intrinsic mechanisms. The hepatic I/R injury model focuses on studying a local sterile inflammatory response driven by reactive oxygen species-related oxidative stress, inflammation, and related signaling pathways like NF-κB pathway [23]. I/R injury is one of the primary causes of early allograft dysfunction and failure after liver transplantation. In contrast, the PH model mimics liver regeneration processes, emphasizing hepatocyte proliferation, signaling pathway activation such as HGF/c-Met and Wnt/β-catenin, and tissue regeneration mechanisms [33]. Taken together, these data still provide evidence that FGF21 has a potential proliferative effect in the liver. Thank you very much for your previous comments.

Minor Concerns

Since FGF21 overexpression approaches were used in the study, I suggest the title of the article change to “FGF21 modulates immunometabolic homeostasis via the ALOX15/15-

HETE axis to aggravate early liver graft injury" or other appropriate writings.

Response: We appreciate your constructive comment. We've changed the title of the article into "FGF21 modulates immunometabolic homeostasis via the ALOX15/15-HETE axis in early liver graft injury". Thank you.

Reviewer #3 (Remarks to the Author):

The authors have assessed the importance of FGF21 in ischemia reperfusion of the liver using a clinical model and experimental murine models. The authors suggest a role of FGF21 in hepatic ischemia reperfusion in particular steatotic livers.

1) Abstract: I found the abstract extremely confusing. Please reorganize including a brief description of the models used.

Response: We have taken your comments seriously and have made the following revisions to the abstract to address your concerns: clarified the research background and objective, simplified the methodology description, highlighted main results and removed redundant information. Moreover, we have included a brief description of the models used in our study. The updated abstract now reads:

Fibroblast growth factor 21 (FGF21) is essential for modulating hepatic homeostasis, but the impact of FGF21 on liver graft injury remains uncertain. Here, we show that high FGF21 levels in liver graft and serum are associated with improved graft function and survival in liver transplantation (LT) recipients. FGF21 deficiency aggravated early graft injury and activated arachidonic acid metabolism and regional inflammation in mouse models of hepatic ischemia/reperfusion (I/R) injury and orthotopic LT. Mechanistically, FGF21 deficiency resulted in abnormal activation of the arachidonate 15-lipoxygenase (ALOX15)/15-hydroxy eicosatetraenoic acid (15-HETE) pathway, which triggered a cascade of innate immunity-dominated pro-inflammatory responses in grafts. Notably, the modulating role of FGF21/ALOX15/15-HETE pathway was even more significant in steatotic livers. In contrast, pharmacological administration of recombinant FGF21 effectively protected against hepatic I/R injury. Overall, our study reveals the regulatory mechanism of FGF21 and offers insights into its clinical application in early liver graft injury after LT.

We hope these revisions improve the clarity of our abstract. Thank you for your constructive comments.

Clinical model:

2) It is unclear what definition of EAD was used. It should be clearly stated in the methods. Is the Olthoff et al used?

Response: Yes, the definition of EAD reported by Olthoff KM, *et al*. [34] was used in our analysis. EAD was defined by the presence of 1 or more of the following variables: (I) total bilirubin (TB) ⩾10 mg/dL on postoperative day 7; (II) an international normalized ratio (INR) ⩾1.6 on postoperative day 7, or (III) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels >2,000 U/L within the first 7 postoperative days. We have added these contents in the manuscript (Materials and Methods section, page 15, lines 24 to 28). Thank you for your comment.

3) It is unclear how you divided patients in two groups, elevated and non-elevated FGF21 serum level.

Response: Thank you very much for your valuable suggestion. These patients were divided into the serum FGF21-elevated group (n = 44) and the non-elevated group (n = 44) according to the median value of change ratio (post-reperfusion/pre-transplant). According to your suggestion, we have emphasized the description in results part and figure legends (Results section, page 6, lines 29 to 30).

4) A correlation between FGF21 serum level and graft survival is suggested, the authors should be cautious in making such correlation, causes of graft failure should be reported.

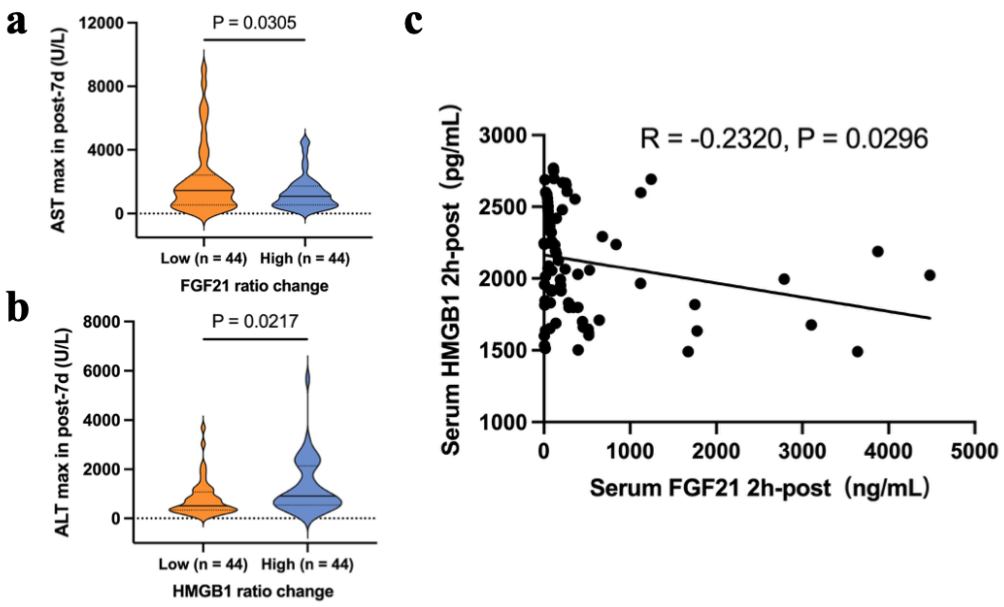
Response: We completely agree with your suggestion. The causes and their corresponding frequencies of Cohort 1 (n = 88) are described as follows: Primary non-function was observed in 5 cases (5.7%); hepatic artery thrombosis occurred in 4 cases (4.5%); biliary complications were noted in 7 cases (8.0%); Chronic rejection was the cause in 9 cases (10.2%). No difference in causes of graft failure was found between serum FGF21-elevated group and the non-elevated group. We have emphasized the description in Supplementary Table S2.

5) Overall the clinical correlation between FGF21 serum level and clinical outcome is quite weak. Would be important to see how FGF21 correlate with other markers of graft function and injury. Is FGF21 just another maker of the severity of I/R injury? Have you assessed other biomolecular markers?

Response: We really appreciate your valuable comments that allowed us to greatly improve this manuscript. After further analysis of our clinical data, we also found that the peak AST level within 7 d after transplantation was decreased in the elevated group (high ratio change of FGF21 serum level) (p = 0.0305, Supplementary Fig. 2a). Overall, the clinical evidence and mechanistic explorations in our study indicated that FGF21 is not only recognized as a

marker of the severity of I/R injury but also implicated in the functional regulation of early liver graft injury. It holds promise as a viable target for therapeutic intervention in clinical settings.

Furthermore, we assessed the association of FGF21 with other biomolecular markers. High mobility group box 1 (HMGB1), an evolutionarily conserved nuclear protein loosely bound to chromatin, is involved in DNA organization and regulation of transcription. When released by damaged cells, extracellular HMGB1 serves as an indicator of cell injury as well as a danger signal stimulating downstream inflammatory reactions via its interaction with sentinel TLR4 and upregulation of NF-κB, which leads to increased production/release of cytokines [35,36]. HMGB1 was also found to have a positive association with peak AST levels within 7 days after transplantation in our present study (p = 0.0217, Supplementary Fig. 2b). Notably, the peripheral FGF21 level was negatively correlated with the peripheral HMGB1 level 2 hours after reperfusion (p = 0.0296, Supplementary Fig. 2c). Thanks again for your suggestions.



Supplementary Fig. S2

6) Regarding the clinical study (results 2.6), I could not find a definition of steatosis, the authors should define how steatosis was defined and assessed.

Response: Thank you for your precious advice. Macrovesicular steatosis (MaS) is an important criterion defining extended-criteria donor organs [37]. MaS was defined as a single vacuole, larger than the nucleus, replacing most of the hepatocyte's cytoplasm and displacing the nucleus toward the cytoplasmic border [38]. Hepatic MaS was characterized quantitatively by the percentage of hepatocytes containing lipid droplets, determined by histological

examination before implanting into recipients. Hepatic steatosis was categorized as follows: no steatosis (MaS affected less than 5% of hepatocytes), mild (MaS affected 5-29% of hepatocytes), moderate (MaS affected 30-60% of hepatocytes) [39]. Microvesicular steatosis (small droplet macrosteatosis) was not included in determining steatosis percentage. According to your suggestions, we have added these contents in the manuscript (Materials and Methods section, page 15, lines 36 and page 16, lines 1 to 8).

7) In general further details regarding the transplant surgery should be included. A standard technique and reperfusion method was used in all recipients?

Response: We appreciate these constructive comments and have revised the paper accordingly. All recipients in our center received routine modified piggyback LT, the general details of the standard technique and reperfusion method is outlined below. (1) Donor livers, procured from donation after brain death or cardiac death with standardized techniques, were perfused with and stored in cold University of Wisconsin (UW) solution. (2) The procedure in a recipient hepatectomy includes hepatic hilum dissection and full division of the perihepatic ligaments, followed by inferior vena cava (IVC) devascularization. (3) Liver grafts were mainly implanted using a modified piggyback technique: 1) Recipient IVC: according to the patient's hepatic vein anatomy, the hepatic veins (left, middle and right) are split from the middle and trimmed into a continuous opening, and the front wall of inferior vena cava is also trimmed longitudinally, and all these together form an inverted triangular incision. 2) Donor IVC: the posterior wall of the donor superior inferior vena cava was cut longitudinally with the two up angers of hepatic superior IVC, also trimmed into an inverted triangular incision. 3) these two inverted triangular incisions are anastomosed. (4) Then, the portal vein anastomosis was completed first, followed by the hepatic artery anastomosis. Reperfusion commenced with the unclamping of the portal vein, allowing portal venous blood flow to the liver, followed by unclamping of the hepatic artery. The condition of the bile ducts should be considered when deciding whether to implant a T-tube. According to your suggestions, we have added these contents in the Supplementary methods section (page 2, lines 2 to 19). Thank you.

8) IS very unclear when the biopsy was taken in steatotic graft, I assume that was taken not more than 30 min after reperfusion? Also is interesting that staining was positive for FGF21 shortly after reperfusion. A comment is necessary.

Response: We apologize for not describing clearly. All biopsies used in our study were pre-transplant biopsies, obtained from the left liver lobe after liver cold storage at back table (before implantation). According to your suggestion, we have emphasized the description when the biopsy was taken in steatotic graft (Materials and Methods section, page 15, lines 15

to 21). Although we didn't detect the FGF21 protein level in post-transplant biopsies, dynamic FGF21 mRNA expression changes between pre- and post-transplantation (within 2 h after reperfusion) in the liver grafts were analyzed using data from the GEO Datasets. Also, we collected serum samples from recipients before transplantation and 2 h after reperfusion to detect the dynamic changes in peripheral FGF21. In the present study, analysis from serum and biopsies both indicated that FGF21 was were markedly increased in the early stage of patients receiving LT. Furthermore, Ye *et al.*'s work also found an increased level of FGF21 protein by IHC in donor liver sections collected after 2 h reperfusion than before implantation [40]. Thank you.

9) Discussion: The authors briefly discussed potential clinical application of FGF21 in steatotic livers. The authors should briefly discuss potential advantage of FGF21 compared to other interventions example defatting during machine perfusion.

Response: Thank you for the insightful comment regarding the discussion of FGF21 in steatotic livers. We have expanded the discussion section to elucidate the potential advantages of FGF21 compared to other interventions, particularly defatting during machine perfusion. We have emphasized FGF21's distinctive mechanisms of action and some comparative advantages concerning efficacy, safety, and feasibility in the manuscript (Discussion section, page 14, lines 25 to 37), outlined as follows:

(1) In addition to alleviating early liver graft injury as reported in our study, the pharmacological administration of FGF21 has also been demonstrated to have multiple beneficial effects in patients with NAFLD/NASH. These benefits include increased hepatic insulin sensitivity, induced fatty acid β-oxidation, inhibition of *de novo* lipogenesis, and decreased delivery of very-low-density lipoprotein to the liver [41,42].

(2) As a water-soluble drug, FGF21 has advantages over most defatting drugs (lipid-soluble ones) due to their better absorption, uniform distribution, efficient elimination, predictable pharmacokinetics, and safer profile, making them preferable for clinical use in terms of efficacy and safety [43,44]. Moreover, the drug characteristics of FGF21 and Fenofibrate were also compared (DrugBank, https://go.drugbank.com/drugs/DB01039) [45,46]:

Response letter Table 1

Parameter	FGF21	Fenofibrate
Half-life	1-2 hours (wild-type); long-acting analogues up to 1-2 weeks	19-27 hours
Solubility	Water-soluble	Lipid-soluble
AUC	Dependent on formulation: e.g., PF-05231023 IV injection AUC	30-50 μg·h/mL after 300mg dose

	is 173.8 ± 25.6 μg·h/mL (3 mg/kg)	
Administration route	Subcutaneous or intravenous injection	Oral administration
Accumulation risk	Low, due to rapid excretion	High, due to prolonged storage in fatty tissues
Metabolism	Primarily metabolized in the liver, excreted through urine	Completely hydrolyzed to fenofibric acid, then metabolized

AUC, Area under the curve; IV, Intravenous injection.

Thanks again for your valuable suggestions, we believe that these modifications will enhance the quality of our manuscript.

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have done an excellent job of revising this manuscript. They have provided a detailed response to the comments from the previous review and have addressed my major concerns. I would like to thank the authors for addressing my initial comments. The manuscript now flows with greater clarity, I enjoyed reading it.

Best regards

Marion Peyrou

Reviewer #2 (Remarks to the Author):

The authors have addressed all of my questions.

Reviewer #3 (Remarks to the Author):

No further comments, authors have replied to all my questions