The arrive guidelines

1. **Title**: Focal Adhesion Kinase-Related Non-Kinase Ameliorates Liver Fibrosis by Inhibiting Aerobic Glycolysis in Hepatic Stellate Cells Via the FAK/Ras/C-Myc/ENO1 Signaling Pathway

2.**Abstract**:

Background: Hyperactivation of hepatic stellate cells (HSCs) is a central link in the development of liver fibrosis. HSCs can perform aerobic glycolysis to provide energy for their activation, and focal adhesion kinase (FAK) can promote aerobic glycolysis ability of cancer cells or fibroblasts, while FAK-related non-kinase (FRNK) can inhibit FAK phosphorylation and its biological function.

Objective: The effect of FRNK on liver fibrosis was observed from the level of aerobic glycolytic metabolism in HSCs.

Mice species: Wild Type (WT) and FRNK knockout ($FRNK^{-/-}$) with the genotype of C57B/6 were used in this study.

Key methods: All mice were obtained intraperitoneal injection with 1.5 μL/g of 10% CCl_4 corn oil solution three times a week to duplicate the liver fibrosis model. Whole liver tissue lysates were extracted to determine the protein level after intervention.

Principle findings: H&E, Masson and Sirius Red demonstrate that the liver fibrosis level of FRNK$^{-/-}$mice is more severe that of WT mice. And its aerobic glycolysis related protein is also increased.

Conclusions: FRNK $^{-/-}$ in mice could promote liver fibrosis and aerobic glycolysis in liver after CCl_4 treated *in vivo*.

Introduction

3. **Background:** Our previous study has verified that the liver fibrosis model in vitro can be duplicated via intraperitoneal injection of CCl_4. [1,2] And many studies used this method to replicate liver fibrosis model in vitro [3]. CCl_4 can lead to pathological change of liver fibrosis, which is similar to almost all the etiologies that can cause liver fibrosis in human. Therefore, we use intraperitoneal injection of CCl4 to conduct our study.

4. **Objectives**: To observe that FRNK knockout in mice could promote liver fibrosis and aerobic glycolysis after the intervention of CCl_4.

Methods

5. Ethic statements: All mouse interventions were approved by the Animal Care Committee (IACUC) of Guizhou Medical University, and

the methods and experimental procedures were performed in accordance with the relevant guidelines and regulations.

6.**Study design**:

a. All the mice were randomly divide into four groups, including WT+Corn, $FRNK^{-/-}$+Corn, WT+CCl_4 and $FRNK^{-/-}$+CCl_4. Each of the group covered 5 mice.

b. All the mice were randomly divided into each group. And each mouse was blindly evaluated its outcome after the intervention.

c. individual mouse was deemed as an experimental unit.

7. **experimental procedures**:

a. Mice were injected intraperitoneally with Corn oil solution containing 10% CCl_4. The mice were sacrificed after respiratory anesthesia with diethyl ether, and the livers of the mice were collected for H&E, Masson and Sirius Red staining, hydroxyproline detection, and Western blot analysis.

B. Mice were injected intraperitoneally three times a week and livers were collected for testing after the fourth week.

C. Test in the cages.

D. Intraperitoneal injection of CCl_4 is a classic modeling method for liver fibrosis, so our study also selected this method for the experiment.

8.animals:

A, b. FRNK$^{-/-}$mice were a gift from the Respiratory and Critical Care Medicine Center, School of Medicine, University of Alabama at Birmingham, USA; Laboratory Animal License No.1801109. WT mice were purchased from the Laboratory Animal Center of Guizhou Medical University. AllexperimentalmicewereonaC57B/6background. All mice tested were healthy males, 8 – 11 weeks old, and weighed 20 ± 3 g. All mice were healthy, had not used any drugs as well as had not participated in any trials. After breeding, mice will be identified as homozygous by tissue PCR.

9.housing and husbandry:

A. All mice were maintained in the normal environment. Select the appropriate cage, bedding, and feed.

B. They were maintained under pathogen-free conditions at controlled temperature (22 ± 2 ° C) and photoperiod (12:12 h light-dark cycle), five per cage, with soft bedding. They were habituated to the above conditions for 2 days before inclusion in the experiment.

10. sample size：

A. A total of 20 mice were used, with 5 animals in each group. Finally, three mice were selected for the test.

B，c. In our previous experience, mice may accidentally die during modeling, so we modeled 5 mice in each group, but the mice did not die in this test. We needed to perform three replicates for each

experiment, so we randomly selected three of the mice for testing.

11.**allocating animal to experimental groups**.

A. Mice of the same genotype were randomly assigned to each corresponding group. For example, 10 WT were randomly assigned to and WT + Corn and WT + CCl_4 groups.

B. Forty-five WT + $CCl_4$and FRNK $^{-/-}$ + CCl_4 5 mice were injected intraperitoneally with Corn solution containing 10% CCl_4 (1.5 uL/g). Five mice in the WT + Corn and FRNK $^{-/-}$ + Corn groups were injected intraperitoneally with WT + CCl_4 and FRNK $^{-/-}$ + CCl4 with the same volume of Corn solution. After the fourth week, mice were anesthetized by inhalation of diethyl ether and then sacrificed, and then liver tissues were collected for the next test. First, we dehydrated and blocked-in paraffin, and then stained the sections with H&E, Masson, and Sirius Red. Then tissue proteins were extracted for hydroxyproline detection and Western blot to detect the relative expression of pY397-FAK, FAK, α-SMA, MCT-1 and ENO1 proteins.

12. **experimental outcomes**:

H&E, Masson and Sirius Red staining indicated that the in vivo liver fibrosis test model was successfully modeled. Detection of hydroxyproline indicated fibrotic content. Simultaneous pY397-FAK and FAK assays indicated that FRNK knockdown prompted increased

FAK phosphorylation, and α-SMA assessed the degree of liver fibrosis from the protein level. MCT-1 and ENO1 can illustrate the level of aerobic glycolysis in mouse liver tissue.

13.**Statistical methods**: Data analysis was performed using SPSS 25.0 software and GraphPad Prism 5.0 for statistical analysis and two-tailed Student's t test for comparison between different groups. $P < 0.05$ was considered statistically significant. Data are presented as mean ± S.D.

Results

14.baseline data:

Group	WT+Corn	FRNK$^{-/-}$+Corn	WT+CCL4	FRNK$^{-/-}$+CCL4
Weight	20g±3g	20g±3g	20g±3g	20g±3g
FRNK Knockout	-	+	-	+
CCL4	-	-	+	+
prior to treatment or testing	-	-	-	-

15.Number analyzed.

a.

WT+Corn	FRNK$^{-/-}$+Corn	WT+CCL4	FRNK$^{-/-}$+CCL4
3/5	3/5	3/4	3/4

B. In the two groups of WT + CCl_4 and FRNK $^{-/-}$ + CCl_4, one animal died during the second modeling at week 4 as well as the third modeling at week 3, respectively. It is possible that the mice died due to fibrosis.

16. **Outcomes and estimation （mean±S.D.)**

Group	WT+Corn	FRNK$^{-/-}$+Corn	WT+CCL4	FRNK$^{-/-}$+CCL4
Relative fibrosis area	1±0.01	1±0.02	10±0.5	19±0.4

Hydroxyproline	200±10	200±10	400±30	750±40
pY397-FAK	1±0.1	1±0.1	1.81±0.1	3.11±0.3
α-SMA	1±0.1	1±0.1	1.91±0.2	2.61±0.2
MCT-1	1±0.1	1±0.1	1.51±0.3	2.41±0.1
ENO1	1±0.1	1±0.1	1.61±0.2	2.31±0.4

17.Adverse events:

a. Mice given CCl_4 developed adverse effects such as emaciation, unresponsiveness and death.

B. These adverse effects were probably due to the development of liver fibrosis in the mouse and we did not modify them because of possible individual differences in response.

Discussion

18. interpretation:

A. Intraperitoneal injection of CCl_4 (1.5 μL/g) into mice for 4 weeks can significantly lead to liver fibrosis, and many previous literatures have also replicated liver fibrosis models by secondary methods, so we selected this classical method. As expected, mouse liver tissues were collected 4 weeks later, and we found that the fibrosis model was successfully replicated by H&E, Masson, and Sirius Red staining, and subsequent hydroxyproline detection in mouse tissues also demonstrated our view. After confirming that the fibrosis model was successfully replicated, we extracted mouse liver tissue proteins, detected the relative expression of the corresponding proteins, and demonstrated the direct relationship between liver fibrosis and aerobic glycolysis in terms of pY397-FAK and glycolysis-related proteins.

B. This study also has some limitations. The purity of CCl_4 reagent also has a certain effect on the replication time of mouse liver fibrosis model. In our study, it was found that there were differences in the purity of CCl4 from different manufacturers and different batches, resulting in differences in the degree of liver fibrosis.

C. We try to follow the principle of 3Rs as much as possible, reduce the use of experimental animals, try to find alternative test models and optimize the principle of test protocol for in vivo testing.

19. In vivo tests can observe the effect of FRNK on liver fibrosis at the macroscopic level, while knockout of FRNK is also easily achieved in animal tests. Therefore, the effect of FRNK on liver fibrosis could be further validated in vitro assays. At the same time, the internal environment in animals is also very similar to that in humans, which can well translate the test results into human biological functions.

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Reference

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