Specific Knockout of Notch-1 in Macrophages Modulate the Progression of Hepatic Insulin Resistance in HFD Fed Mice *via* Regulating IRE1α-XBP1 Signals

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ABSTRACT

Objective: To develop an intervention based on Notch-1 signalling pathway blockade by investigating the potential application of the neurogenic locus notch homologue protein 1(Notch-1) signalling pathway as a key regulator of chronic inflammation and adipogenesis in the treatment of hepatic insulin resistance (HIR).

Study Design: Experimental study.

Place and Duration of the Study: Animal Laboratory of the Fourth Hospital of Hebei Medical University, Shijiazhuang, China, from April 2021 to June 2022.

Methodology: HIR models were established in Notch-1^{WT} and Notch-1$^{MAC\text{-}KO}$ mice by high fat diet (HFD) for 16 weeks. Haematoxylin and eosin (HE) staining and oil red O (ORO) staining were used to detect inflammatory infiltration and lipid accumulation in each group. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of TNF-α and IL-6. Free fatty acid (FFA) and total cholesterol (TC) were measured with relevant kits. Moreover, real-time quantitative polymerase chain reaction (PCR) was performed to detect the relative expressions of F4/80, Mcp1, and CD11b in hepatic tissues. Mass spectrometry was used to analyse the levels of triglyceride (TG), diacylglycerol (DAG) and conformite europeenne (CE) in liver tissue. Western blotting was used to detect the expression of related proteins.

Results: Specific knockdown of Notch-1 in macrophages decreases the relative fluorescence intensity of CD68 and attenuates inflammatory infiltration and lipid degeneration. There was no difference in plasma levels of FFA and TG. Specific knockdown of Notch-1 in macrophages decreases the expression of F4/80, Mcp1, and CD11b, as well as the levels of TG, DAG, CE, IL-6, and TNF-α.

Conclusion: Specific knockout of Notch-1 in macrophages may reduce HIR by inhibiting the IRE1α-XBP1 signalling pathway.

Key Words: *Hepatic insulin resistance, Macrophages, Notch-1, IRE1α, XBP1.*

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INTRODUCTION

Currently, obesity is recognised as a pathology characterised by anomalous fat deposition within the body, leading to severe health consequences. It stands as a principal contributor to hepatic insulin resistance (HIR), with its mechanisms significantly implicated in the aetiology of HIR.[1-3] HIR is primarily characterised by an impaired ability of insulin to inhibit glucose output. Additionally, obesity places a substantial health burden on various human organ systems, resulting in health disorders such as nonalcoholic fatty liver disease (NAFLD), insulin resistance (IR), and type 2 diabetes mellitus (T2DM).[4,5]

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IR also serves as a pathophysiological basis of insulin resistance syndrome (IRS) and is closely associated with lipid peroxidation, mitochondrial insufficiency, structural and functional damage to cell tissues, and altered energy homeostasis.[6,7] Obesity precipitates the onset and progression of HIR, as evidenced by hepatic fat accumulation and enhanced gluconeogenesis. As hepatic insulin dysfunction progresses, IR becomes apparent in the liver, characterised by the reduced capability of insulin to curtail glucose production, culminating in gluconeogenesis.[8] Thus, the pathogenesis of HIR and T2DM is similar and leads to endoplasmic reticulum stress (ERS) and associated inflammasome activation.[9,10]

Recent investigations have elucidated that the Notch-1 signalling pathway may modulate insulin signalling and cellular operations through various mechanisms, such as modulating the expression and functionality of insulin receptors, governing glycogen synthesis and metabolism, and managing the synthesis and oxidation of fatty acids. As demonstrated by increasing studies, the Notch-1 signalling pathway is closely associated

with adipogenesis and inflammation.[11-13] Therefore, it is hypothesised that Notch-1 may be a potential treatment target for HIR, but its molecular mechanism and role remain unclear. The aim of this study was to determine the effect of specific knockout of Notch-1 in macrophages on HIR.

METHODOLOGY

This experimental study was conducted at the Animal Laboratory of the Fourth Hospital of Hebei Medical University, Shijiazhuang, China, from April 2021 to June 2022. C57BL/6 wild-type and myeloid-specific Notch-1-knockout (Notch-1MAC-KO) mice were purchased and generated, with each group consisting of 6 mice. Mice were housed under a 12-hour light-dark cycle, fed a high-fat diet for 6 weeks to induce hepatic insulin resistance (HIR), and monitored *via* intraperitoneal glucose tolerance tests (IPGTT). Blood samples were collected from the tail vein and frozen at -70°C. Mice were first euthanised with 20% isoflurane, and the liver was soaked in 75% alcohol for 3 seconds before collecting and removing unwanted tissue. Liver tissue was cut, ground, and trypsinised, filtered, and centrifuged to isolate the cells. The washed cells were adjusted to a specific concentration and transferred to a culture plate for culture. This research experiment conforms to the relevant regulations of national laboratory animal ethics and has been reviewed by the Laboratory Animal Ethics Committee of the Fourth Hospital. Liver tissue samples were homogenised with QIAGEN tissue lysis reagent in methanol-water. Lipid extracts were analysed using an AB Sciex QTRAP 5500 mass spectrometer combined with ultra-performance liquid chromatography (UPLC) tandem mass spectrometry. The sections were then stained with the HE staining and oil red O (ORO) staining, respectively. Finally, liver histology was performed under an inverted fluorescence microscope and quantified using Image Pro Plus 6.0.

The hepatocytes harvested from each group were fixed with paraformaldehyde, permeabilised for 5 minutes, washed with PBS for 5 minutes, and blocked with blocking buffer for 30 minutes, followed by incubation with primary antibodies at room temperature for 1 hour. Then they were rinsed 3 times with PBST, incubated at room temperature away from light for 1 hour, rinsed 3 times with phosphate buffered saline with tween (PBST), and blocked. Finally, they were quantitatively and morphologically analysed using laser scanning confocal microscopy.

Blood drawn from the tail vein was centrifuged at 15,000g to isolate the plasma. Then the plasma free fatty acid (FFA) and total cholesterol (TC) were detected using commercially available kits and photochemistry.

Hepatic tissue samples were harvested, immediately frozen in liquid nitrogen, and homogenised in TRIzol reagent (Invitrogen). As previously described, total RNA was extracted using the RNeasy mini kit (QIAGEN Inc). Gene expression primers and probe sequences were designed using Primer Express or purchased from Applied Biosystems, Inc. (Thermo Fisher Scientific), and real-time quantitative PCR was performed using the Bioline SensiFAST Probe One Step Kit. EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80): F: 5'-GCATACTGTCTGTGGCTGTAATCG-3'; R: 5'-GTGGATGTAGGTGTTCTGGTTTCG-3'; Monocyte chemoattractant protein-1 (Mcp1): F: 5'-TGCCCTAAGGTCTTCAGCAC-3'; R: 5'-AAGGCATCACAGTCCGAGTC-3'; Integrin alpha M (CD11b): F: 5'-CCATGACCTTCCAAGAGAATGC-3'; R: 5'-ACCGGCTTGTGCTGTAGTC-3'.

Collected the cell supernatant, centrifuged at 12,000 rpm for 10 min at 4°C, and measure the concentrations of inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) with an ELISA kit (Novus Biologicals, CO, USA or Abcam, Cambridge, UK). All samples were taken in triplicate and measured at a wavelength of 450 nm.

HepG2 cells were cultured under specific conditions before being co-cultured with peritoneal macrophages from Notch-1WT and Notch-1MAC-KO groups in transwell chambers. These macrophages were also treated with palmitic acid and/or an IRE1α activator, HY-139212, before co-culture. Post-treatment, the macrophages underwent flow sorting, and differently treated HepG2 cells were organised into four groups (WT, KO, WT+HY-139212, and KO+HY-139212) for further analysis.

Liver tissue and cells were lysed using radio-immunoprecipitation assay (RIPA). The total protein was obtained by centrifugation at 12,000 g and 4°C for 40 minutes and measured using the BCA Protein Assay Kit (Beyotime). Subsequently, a supernatant aliquot (40-80 μg/well) was separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane, followed by incubation with the antibodies against phospho-Insulin Receptor (p-IR), phospho-Insulin Receptor (p-IR), phospho-Insulin Receptor Substrate-1 (p-IRS-1), Notch-1, phospho-inositol requires kinase enzyme 1α(p-IRE1α), X-box binding protein 1 (XBP1), glucose-regulated protein 78 (GRP78), GRP94, IL-6 and GAPDH (Abacm). After washing with TBS containing 0.05% TBST, the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies for 2 hours. Finally, the protein bands were visualised with chemiluminescence, and the blot density was analysed with image.

All results were expressed as mean ± standard deviation by SPSS 20.0. One-way ANOVA was used for differences among groups, and Student's t-test was used for comparison between two groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

More CD68-positive macrophages were found by immunofluorescent staining in Notch-1^{WT} group than in Notch-$1^{MAC\text{-}KO}$ group as shown in Figure 1 (A and B). To investigate the effect of specific knockout of Notch-1 on inflammatory infiltration and lipid metabolism in hepatic tissues in HFD-induced HIR, ORO staining and HE staining was performed. It was observed that inflammatory infiltration and lipid accumulation were significantly attenuated in HFD-fed mice in Notch-$1^{MAC\text{-}KO}$ group as compared to those in Notch-1^{WT} group as shown in Figure 2 (A and B).

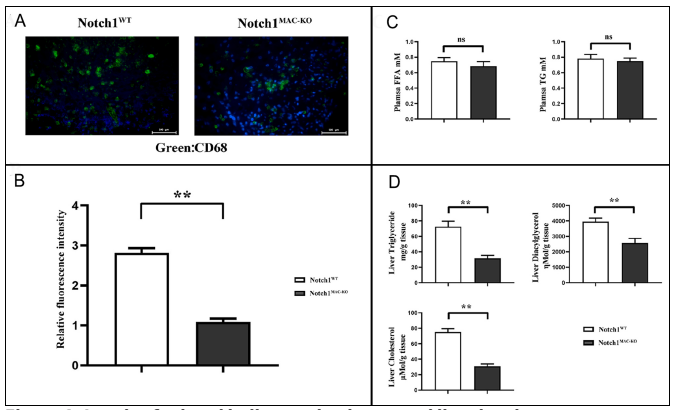

Figure 1: Levels of related indicators in plasma and liver in mice.
A: Immunofluorescent staining results of mice in Notch1^{WT} group and mice in Notch1^{MAC-KO} group, green representing CD68. B: The relative fluorescence intensity of CD68 in Notch1^{WT} group and Notch1^{MAC-KO} group was significantly reduced. C: Plasma FFA and TG. D: Levels of TG, DAG and CE in the liver. Notch-1^{MAC-KO} group had significantly decreased levels of TG, DAG and CE compared with Notch-1^{WT} group: p<0.01, ns: p>0.05, n=6/group.**

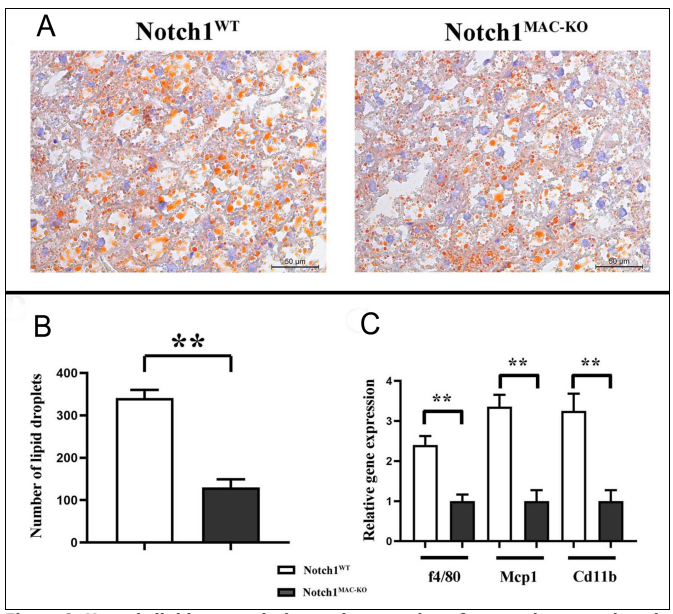

Figure 2: Hepatic lipid accumulation and expression of macrophage markers in mice.
A: Representative oil red O-stained and HE-stained hepatic tissue sections in Notch-1^{WT} group and Notch-1^{MAC-KO} group. B: Significantly reduced lipid accumulation in Notch-1^{MAC-KO} group as compared to Notch-1^{WT} group. C: Expressions of F4/80, Mcp1, and CD11b in the liver of mice in Notch-1^{WT} group and Notch-1^{MAC-KO} group. **: p<0.01, ns: p>0.05, n=6/group.

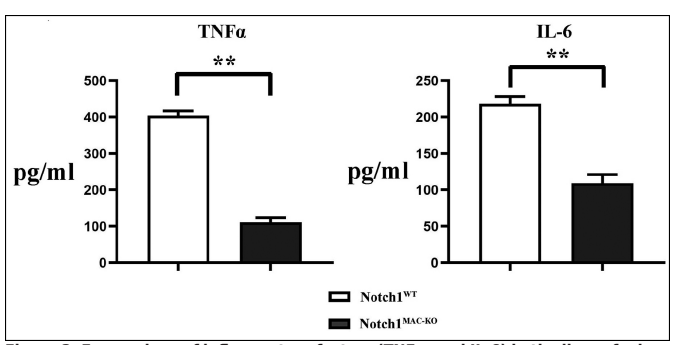

Figure 3: Expressions of inflammatory factors (TNF-α and IL-6) in the liver of mice. The results of ELISA showed that the expressions of TNF-α and IL-6 were significantly lower in Notch-1^{MAC-KO} group than those in Notch-1^{WT} group. **: p<0.01, n=6/-group.

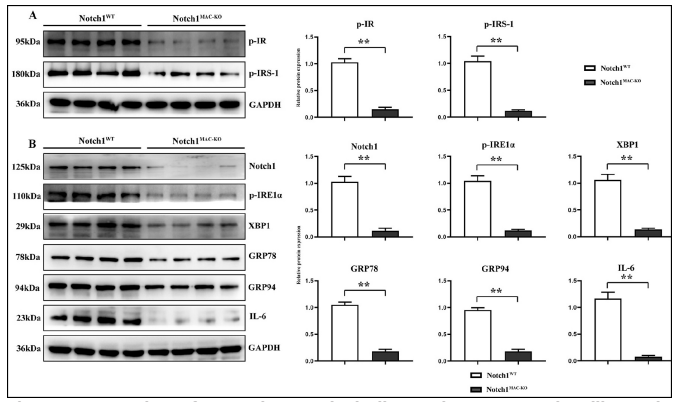

Figure 4: Expressions of p-IR and p-IRS-1 in the liver and IRE1α-XBP1 signalling pathway-related proteins in hepatic macrophages.
A: Protein bands of p-IR and p-IRS-1 in the liver and Notch-1, p-IRE1α, XBP1, GRP78, GRP94, and IL-6 in hepatic macrophages. The four channels per group indicated four representative mice from which the relevant proteins were extracted. B: Relative protein expressions of p-IR and p-IRS-1 in the liver and Notch-1, p-IRE1α, XBP1, GRP78, GRP94, and IL-6 in hepatic macrophages. **: p<0.01, n=4/group.

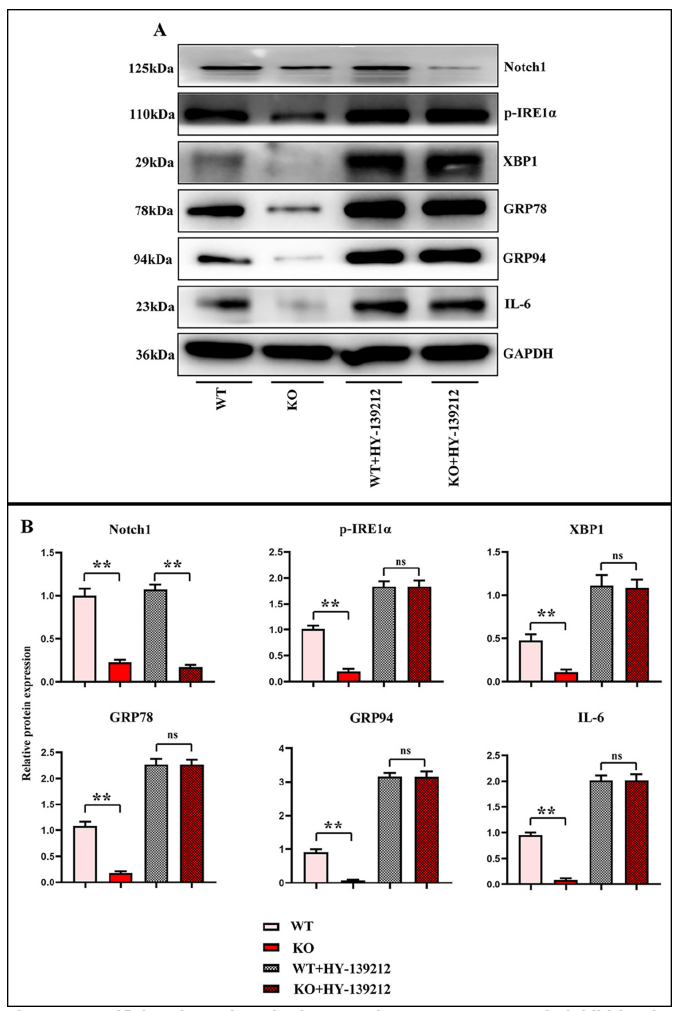

Figure 5: Specific knockout of Notch-1 in macrophages attenuates HIR by inhibiting the IRE1α-XBP1 signalling pathway.
A: Protein bands of Notch-1, p-IRE1α, XBP1, GRP78, GRP94, and IL-6. B: Relative protein expressions of Notch-1, p-IRE1α, XBP1, GRP78, GRP94, and IL-6. **: p < 0.01, n=3/group.
Specific knockdown of Notch-1 in macrophages can inhibit the progression of hepatic insulin resistance in mice. Specific knockdown of Notch-1 in macrophages inhibits IRS phosphorylation and by regulating IRE1α-XBP1 signalling, the expression of inflammatory factors was inhibited, thereby inhibiting the progression of insulin resistance in the liver of HFD-fed mice.

Obvious up-regulation of lipid degeneration- and inflammatory infiltration-related indicators was found in Notch-1^{WT} mice. The changes in the levels of related indicators in hepatic tissues and plasma were explored by specific knockout of Notch-1. The results showed that the levels of plasma FFA and TG had no differences between Notch-1^{MAC-KO} group and Notch-1^{WT} group. Notch-1^{MAC-KO} group had significantly lower levels of TG, DAG and CE in hepatic tissues than Notch-1^{WT} group ($p < 0.05$) as shown in Figure 1 (C and D). Besides, it was found that the relative expressions of F4/80, Mcp1 and CD11b in Notch-1^{MAC-KO} group were significantly lower than those in Notch-1^{WT} group ($p < 0.05$) as shown in Figure 2C. Moreover, the relative fluorescence intensity of macrophage CD68 was detected in the two groups by immunofluorescent staining. The relative fluorescence intensity in Notch-1^{WT} group was significantly higher than that in Notch-1^{MAC-KO} group. It could be inferred that specific knockout of Notch-1 will significantly reduce the hepatic lipid content in mice, suggesting that Notch-1 contributes to hepatic lipid degeneration.

The expressions of inflammatory factors TNF-α and IL-6 were detected by ELISA in Notch-1^{WT} and Notch-1^{MAC-KO} groups. The results revealed that the levels of IL-6 and TNF-α in hepatic tissues in the Notch-1^{MAC-KO} group were significantly lower than those in the Notch-1^{WT} group, indicating that specific knockout of Notch-1 significantly reduces hepatic inflammatory markers as shown in Figure 3.

It was found by Western blotting that the relative protein expressions of p-IR, p-IRS-1, Notch-1, p-IRE1α, XBP1, GRP78, GRP94 and IL-6 in hepatic tissues in Notch-1^{MAC-KO} group were significantly lower than those in Notch-1^{WT} group ($p < 0.05$) as shown in Figure 4 (A and B).

In vitro experiments revealed that the expressions of Notch-1, p-IRE1α, XBP1, GRP78, GRP94, and IL-6 significantly declined in KO group as compared to those in the WT group ($p < 0.05$), and the expression of Notch-1 was significantly lower in KO+HY-139212 group than that in WT+HY-139212 group. After the IRE1α activator HY-139212 was added, the relative protein expressions of Notch-1, p-IRE1α, XBP1, GRP78, GRP94, and IL-6 significantly rose in WT group and KO group without differences between the two groups ($p > 0.05$) as shown in Figure 5 (A and B).

DISCUSSION

Obesity symbolises a quintessential ailment of contemporary civilisation, aligning as one of the quintet of pre-eminent healthcare and social crises globally, alongside HIV/AIDS, drug addiction and alcoholism, affecting more than a billion people globally.[14,15] Research has determined that alterations in body weight can either boost or impair insulin sensitivity, with obesity and insulin resistance being interconnected; specifically, the excessive build-up of fat tissue triggers systemic insulin resistance, encompassing endocrine dysfunctions and inflammation.[16,17] HIR refers to a decrease in the cellular effect of the hepatic insulin unit concentration, i.e., a decrease in the sensitivity of tissues to insulin.

Notch-1, a substantial 300 kDa type 1 transmembrane glycoprotein and member of the Notch homologue cohort implicated in ontogenesis, exhibits a structure hallmarked by extracellular domains with numerous epidermal growth factor (EGF)-like repetitions and an intricate intracellular domain variety. The Notch-1 pathway is associated with cell differentiation, proliferation, apoptosis, and adhesion as well as adipogenesis and inflammatory infiltration.[18] Studies have found that the Notch-1 signalling pathway can ameliorate fatty liver and insulin resistance.[19] In this study, therefore, the wild-type and specific Notch-1-knockout mice were generated, and they were fed with HFD to establish HIR models.

It was observed by HE staining and ORO staining that specific knockout of Notch-1 could reduce HIR-induced lipid accumulation and inflammatory infiltration in hepatic tissues. A close association between Notch-1 and hepatic lipid accumulation was found, i.e., specific knockout of Notch-1 could lower the levels of TG, DAG, and CE in HIR models. However, no impact of specific knockout of Notch-1 on plasma FFA and TG was observed in the two groups. It suggested that specific knockout of Notch-1 in mice has no impact on FFA and TG in plasma. Moreover, the results of ELISA showed that specific knockout of Notch-1 had a significant inhibitory effect on inflammatory factors in the liver.

Recent academic insights suggest that stress in the endoplasmic reticulum can initiate insulin resistance in peripheral organs, such as the liver, muscle, and fat tissue, by activating the inositol-requiring enzyme 1α, thereby severely affecting pancreatic beta-cell health and peripheral insulin action in type 2 diabetes mellitus.[20,21] The macrophage CD68 in hepatic tissues was extracted by flow sorting, and then the relative protein expressions of Notch-1, p-IRE1α, XBP1, GRP78, GRP94, and IL-6 in CD68 and of p-IR and p-IRS-1 in hepatic tissues were detected using Western blotting. The results manifested that these proteins were all significantly down-regulated in hepatic macrophages and tissues after specific knockout of Notch-1. In addition, the IRE1α activator was added to the HIR model in the *in vitro* experiment to verify the results of the *in vivo* experiment. Finally, it was concluded that specific knockout of Notch-1 in macrophages exerts a protective effect against HIR by inhibiting the expression of IRE1α-XBP1 signalling pathway-related proteins. It has been found that FoxO1 and Notch1 coordinate the regulation of hepatic glucose metabolism. Combined haplotype insufficiency of FoxO1 and Notch1 significantly improves insulin sensitivity in diet-induced insulin resistance, and Notch1 function gains promote insulin resistance and induce glucose-6-phosphatase expression in a FoxO1-dependent manner. Pharmacological blockade of Notch signalling with γ-secretase inhibitors improves insulin sensitivity after administration in both lean and obese, insulin-resistant mice. Notch inhibition is beneficial for diabetes treatment, in part because it helps counteract FoxO1-driven excess

hepatic glucose production.[22,23] But corresponding clinical trials are needed to verify and explain this conclusion.

CONCLUSION

Specific knockout of Notch-1 can ameliorate HIR and suppress the expressions of TG, DAG and CE in HIR models, which may be related to the inhibition of IRE1α-XBP1 signalling pathway-related proteins. These findings provide new ideas for the treatment of HIR.

ETHICAL APPROVAL:

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Experimental Animal Ethics Committee of the Fourth Hospital of Hebei Medical University (LACUC-4th Hos Hebmu).

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

WZ, QG, SR: Built animal models and manuscript writing.
QG, YL, YW: Conceived the study.
WZ, LF: Carried out the statistical analysis.
All authors approved the final version of the manuscript to be published.

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