Liver knockout YAP gene improved insulin resistance-induced hepatic fibrosis

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Abstract

Yes-associated protein (YAP), as a co-activator of transcription factors, is a downstream protein in the Hippo signaling pathway with important functions in cell proliferation, apoptosis, invasion and migration. YAP also plays a key role in the development of CCl₄-induced liver fibrosis. However, the mechanism of YAP during hepatic fibrosis progression and reversion is still unclear. Mild liver fibrosis was developed after 4 months of high-fat diet (HFD) stimulation, and we found that the YAP signaling pathway was activated. Here, we aim to reveal whether specific knockout of Yap gene in the liver can improve liver fibrosis induced by insulin resistance (IR) stimulated by HFD, and further explain its specific mechanism. We found that liver-specific Yap gene knockout improved IR-induced liver fibrosis and liver dysfunction, and this mechanism is related to the inhibition of the insulin signal pathway at the FoxO1 level. These findings provide a new insight, and Yap is expected to be a new target to reverse the early stage of liver fibrosis induced by IR.

Key Words

- YAP
- FoxO1
- liver fibrosis
- insulin resistance

Introduction

Liver fibrosis is characterized by excessive deposition of extracellular matrix (ECM), which is caused by chronic liver injury (Hui & Friedman 2004) due to schistosomiasis and chronic viral hepatitis infection, non-alcoholic fatty liver disease (NAFLD), ALD, cholestatic and autoimmune liver disease (Poelstra 2016, Weiskirchen & Tacke 2016). Hepatic stellate cell (HSC) plays an important role in the occurrence and development of hepatic fibrosis. The resting HSC is located in the subendothelial space of Disse and has cytoplasmic lipid droplets as a repository of vitamin A (Geerts et al. 2001, Satapathy & Sanyal 2015). When injury occurs, resting HSCs may undergo trans-differentiation from vitamin A storage cells to myofibroblast-like cells and are activated. Activated HSCs express α-smooth muscle actin (α-SMA), release excessive ECMs, including collagen and fibronectin, which play a dominant role in progressive liver fibrosis (Bataller & Brenner 2005, Friedman 2008a,b). Therefore, revealing the molecular mechanism that drives ECM deposition in the early stages of HSC activation is one of the key measures to prevent and reverse the fibrosis response. However, the molecular mechanism of HSC activation
has not been clarified, and so far, there are no specific drugs for reversing early stage liver fibrosis. Recently, the transcriptional coactivator Yes-associated protein (Yap) was found to be activated by interfering with HSC, which could potentially reverse hepatic fibrosis (Mannaerts et al. 2015).

As a coactivator of transcription factors, YAP promotes cell proliferation and inhibits apoptosis through the interaction between its transcriptional coactivator activity and TEAD (transcriptional enhanced associate domain) in the Hippo signaling pathway (Huang et al. 2005, Pan et al. 2010). In the mammalian liver, the Hippo signaling pathway is involved in pathological processes such as development and regeneration, cancer and fibrosis (Pan et al. 2010, Mannaerts et al. 2015). In the process of CCl₄-induced liver fibrosis in mice, YAP was translocated from the cytosol into the nucleus in HSC and resulted in the activation of HSC. If YAP was inhibited by gene knockdown or by the YAP inhibitor vitipofen (VP), HSC activation and fibrosis can be prevented (Mannaerts et al. 2015, Yu et al. 2019). The YAP translocation and the activation of HSC were related to the upregulation of some genes involved in matrix remodeling, actin cytoskeleton, cell proliferation and immunity. Among them, the strongest upregulated genes are the target genes of Yap, such as Ankrd1 (recombinant ankyrin repeat domain protein 1) and CTGF (connective tissue growth factor). Ankrd1 was the most induced gene expression to mediate TGF-β signal in response to injury in heart and muscle (Kojic et al. 2011), and TGF-β was the most powerful fiber-forming medium which induced activation of PYK2-Src-RhoA triad leading to YAP/TAZ activation for CTGF induction in liver fibrosis (Kim et al. 2020). CTGF is a cysteine-rich ECM protein, which plays an important role in tissue remodeling and wound repair by the induction of some fiber genes expression, such as fibronectin, collagens (I, III, IV and VI), and some genes, such as αVβ 3 and α5β 1 integrins, to bind with cell surface receptors (Lau 2016). Additionally, CTGF also promoted the activation and proliferation of HSC (Huang & Brigstock 2012). More importantly, both Ankrd1 and CTGF were upregulated earlier than Acta2 (actin alpha 2, also named α-SMA) (Mannaerts et al. 2015), which means that YAP regulated the early stage of HSC activation. Therefore, inhibition of YAP is likely to be a new way to prevent liver fibrosis or reverse its progression. However, if Yap is involved in liver fibrosis induced by insulin resistance (IR) has not been reported.

In this study, mouse liver fibrosis was induced by IR using HFD stimulation, and the liver YAP pathway and insulin pathway were tested and analyzed. Therefore, YAP gene induced a specific knockout in mice liver, and whether liver fibrosis was improved will be further evaluated and the related mechanisms will be discussed.

Materials and methods

Animals

Floxed Yap mice (Yaplox/lox), albumin-Cre, and liver-specific YAP gene knockout (L-YKO) mice (albumin Cre::YAPlox/lox) have been previously described (Schlegelmilch et al. 2011). In this experiment, 2-month-old L-YKO mice were used. The control group (CNTR) mice were Yaplox/lox. All mice were housed under standard laboratory conditions (12 h light:12 h darkness cycle) and defined environmental conditions (temperature: 22°C, humidity: 65–70%) with food and water available ad libitum in the SPF barrier laboratory of the Experimental Animal Center of North China University of Science and Technology. Mice were fed with normal maintenance feed or HFD (60% kcal from fat, 20% from carbohydrates and 20% from protein, purchased from Shuyi Shuer Biotechnology Co., D12492i). This experiment was complied with the ethical requirements of laboratory animals in China and was approved in accordance with the ‘Institutional Animal Care and Use Committee (IACUC)’ of the People’s Republic of China.

Blood biochemistry analysis

Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were analyzed to evaluate liver functions using commercial kits (Beijing Ruizheng Shanda Biological Engineering Technology Co. Ltd., China) by an automatic biochemical analyzer (Beijing Prang New Technology Co. Ltd., China).

Histological analysis and Masson trichrome staining

To assess the general morphology of the liver, the liver was stored in 4% paraformaldehyde for more than 12 h; then the tissues were processed routinely for paraffin embedding, and 4–5 µm thick sections were cut and placed on glass slides. The paraffin-embedded sections were dewaxed with xylene, washed by gradient ethanol to water, incubated with hematoxylin and eosin (H&E, Beijing Leagene Biotechnology Co. Ltd., China) for 5 min, and sealed after conventional ethanol dehydration. Finally, sections were analyzed under a Nikon light microscope at the indicated magnification.
To assess the severity of liver fibrosis, Masson trichrome staining was performed to visualize the fibrosis. The upper liver paraffin-embedded sections were dewaxed and dealt with as previously described according to the kit’s instructions (Beijing Leagene Biotech Co. Ltd.) (Zhang et al. 2012).

**Glucose tolerance tests (GTT) and insulin tolerance tests (ITT)**

**GTT:** After fasting for 12 h, mice received i.p. injections of glucose (1.5 g/kg), and the blood glucose level was detected at different time points (0, 15, 30, 60, 120 min) after glucose administration. The areas under the curve (AUC) were calculated using trapezoidal integration (Jia et al. 2014, Yao et al. 2017).

**ITT:** After fasting for 4 h, mice received i.p. injections of insulin (0.8 U/kg), and the blood glucose level was detected at different time points (0, 15, 30, 60, 120 min) after insulin administration. The areas under the curve (AUC) were calculated using trapezoidal integration (Chen et al. 2020a).

**Immunohistochemistry**

Immunohistochemistry was done for determining the expression of p-FoxO1, FoxO1, YAP, α-SMA. Briefly, 5 µm sections were deparaffinized with xylene and rehydrated in ethanol series. The sections were treated with 3% hydrogen peroxide to inactivate the endogenous peroxidase activity and then immersed in citrate buffer at 95°C for 20 min. After blocking with 10% goat serum, tissue sections were incubated with primary antibodies against p-FoxO1 (1:100), FoxO1 (1:100), YAP (1:200), α-SMA (1:200) at 4°C overnight (the antibodies are shown in Table 1). On the second day, after washing with PBS, the tissues were incubated with biotinylated anti-rabbit link secondary antibody for 30 min and with DAB for the coloring substrate, and then counterstained with Mayer’s hematoxylin.

**Protein analysis and Western blot analysis**

Liver total proteins were extracted and the concentrations were quantified using Bradford (Bio-Rad) assay. The proteins were separated by electrophoresis and transferred to PVDF membranes, which were blocked for 60 min in TBST containing fat-free milk. The membranes were then incubated overnight at 4°C using the following primary antibodies: IRS-1, IRS-2, Akt, p-Akt, p-FoxO1, FoxO1, Collagen1, YAP, p-YAP, α-SMA and TGF-β1 (the antibodies are shown in Table 1).

Subsequently, Western blot was carried out according to the standard protocol. The immunoreactive bands were visualized using the ECL kit according to the manufacturer’s instructions (Beijing Zoman International Bio-Gene Technology Co. Ltd., China). The signal intensities of the target proteins were analyzed by a gel imager (Bio-Rad) and the grey values were analyzed by Image J software. The housekeeping protein α-tubulin served as an internal control.

**RNA extraction and real-time PCR**

The total RNA was extracted with Trizol (Ambion) and reversely transcribed into cDNA using the a kit (Promega). Subsequently, real-time PCR was performed with a PCR instrument (Takara Corporation). PCR amplification was carried out using the specific primers which were synthesized by the company: YAP, Collagen1, MMP2, MMP9 and TGF-β1. β-actin was used as the housekeeping gene as the control for normalization (Chen et al. 2020b). The PCR primers are listed in Table 2.

**Cell culture and primary hepatocyte isolation**

JS-1 cell line (hepatic stellate cell line) was purchased from Hunan Feng-hui Biotechnology Co. Ltd. and was cultured in a complete medium: DMEM was supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/mL of penicillin, and 100 units/mL of streptomycin.

Mouse primary hepatocytes were isolated from 8-week-old mice with a protocol previously described and cultured in DMEM complete medium (Zhang et al. 2012).

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**Table 1** Details of the antibodies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Company</th>
<th>Concentration</th>
<th>Clonality</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS-1</td>
<td>Affinity</td>
<td>1:750</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>IRS-2</td>
<td>Affinity</td>
<td>1:2000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Akt</td>
<td>EPIJ MICS</td>
<td>1:2000</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>p-Akt</td>
<td>Arigo</td>
<td>1:250</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>p-FoxO1</td>
<td>Cell Signaling Technology</td>
<td>1:500</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>FoxO1</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Collagen1</td>
<td>Affinity</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>YAP</td>
<td>Affinity</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>p-YAP</td>
<td>Affinity</td>
<td>1:1000</td>
<td>Polyclonal</td>
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<tr>
<td>α-SMA</td>
<td>Affinity</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>Polyclonal</td>
</tr>
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</table>

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Recombinant lentivirus preparation and cell transient transfection

The cDNA encoding human YAP sequence was amplified by RT-PCR with the primer: sense 5'-TGCTATCGAATGGATCCCGGACAGCGC-3' and antisense 5'-TTGCGGCCGCTATAACCATGTAAAGCTTTCT-3'. And then the DNA fragment was inserted into pCDH-CMV-MCS-EF1-Puro by the XbaI/NotI site to produce the plasmid named pCDH-CMV-hYAP-GFP.

The HEK-293T cell line was used as a host for virus packaging. The pCDH-CMV-hYAP-GFP or pCDH-CMV-GFP (empty lentiviral plasmids, YouBio, China) was mixed along with the psPAX2 and pMD2.G plasmids, respectively, and was co-transfected into HEK-293T cells in a six-well palate using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s protocol to get overexpression of YAP gene virus (pLenti-YAP-wt) and empty virus (pLenti-em), respectively. Virus particles were harvested after 72 h post-transfection and the viral titration was calculated. Cells were cultured until approximately 80% confluent and were transfected using produced virus particles at a multiplicity of infection (MOI) of 50 in the presence of 10 µg/mL polybrene (Sigma).

Two culture wells were randomly selected as the control group and were transfected with pLenti-em virus particles, and the other two culture wells were randomly selected and were transfected with pLenti-YAP-wt virus particles. After transfection for 24 h, all the cells were collected and used for RNA and protein extraction.

Statistical analysis

All results were presented as mean ± s.d., and P values were determined by one-way ANOVA. For comparison between the two groups, the Dunett t-test was used. P < 0.05 was considered significant, and P < 0.01 was considered extremely significant. All statistical analyses were performed using SPSS18.0 software (SPSS).

Table 2  Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2</td>
<td>5'-CAACGGTCCGGGAATACACCGACGAG-3'</td>
<td>5'-CCAGAAAGGTGAAAGGGAAGAAGA-3'</td>
</tr>
<tr>
<td>MMP9</td>
<td>5'-GCTGACTAGTAAAGGACGCGG-3'</td>
<td>5'-AGGAACAGGAAGGGAAGAAGG-3'</td>
</tr>
<tr>
<td>YAP</td>
<td>5'-GACCTCTGTTTTCGCCATGAA-3'</td>
<td>5'-ATTGTTCTAATCTCTGAGACG-3'</td>
</tr>
<tr>
<td>FoxO1</td>
<td>5'-CCTCATGACGCTGATT-3'</td>
<td>5'-CACCGATCTCCTACATGCC-3'</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5'-TGGAATACAGGGGATCG-3'</td>
<td>5'-ACTTCAAACAGGGTCTCTC-3'</td>
</tr>
<tr>
<td>CTGF</td>
<td>5'-CAACGGTCCGGGAATACACCGACGAG-3'</td>
<td>5'-TTAGGCGCTTACATGTAC-3'</td>
</tr>
<tr>
<td>Ankrd1</td>
<td>5'-CCAGAAAGGTGAAAGGGAAGAAGA-3'</td>
<td>5'-GAACCTCGGACACATCCAC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CTAAGGCCAACCGTGAAAAG-3'</td>
<td>5'-ACCAGGGCATAACGGGAC-3'</td>
</tr>
</tbody>
</table>

Results

Mice exhibited abnormal glucose metabolism and mild liver fibrosis after HFD stimulation

2-month-old CNTR mice were selected and given normal and high-fat diet feed for 4 months. Compared with the CNTR group, body weight and liver mass of CNTR+HFD group had significantly increased after 4 months HFD treatment (Fig. 1A and B). ALT and AST in the serum of the CNTR+HFD group, the indicators of liver injury, increased significantly compared with that of the CNTR group (Fig. 1C and D), which indicated that liver function was damaged. Mice liver morphological features were further observed by H&E staining. In the CNTR+HFD group, the hepatocytes were swollen, the connections between cells were loose, and lipid droplets of different sizes could be seen in the visual field (Fig. 1E). Fibrosis could not be detected in CNTR mice liver using Masson staining, and mild fibrosis appeared around the portal area of the liver in CNTR+HFD mice (Fig. 1F). Moreover, the expression of YAP increased significantly in HFD-stimulated liver compared with the control liver, and YAP was mainly expressed around the portal area and translocated from the cytosol into the nucleus (Fig. 1G).

The expression of fibrosis-related genes and proteins increased in normal mice after 4 months of HFD stimulation

Since the liver is the main organ involved in glucose metabolism, we further conducted GTT and ITT to test the whole-body glucose metabolism situation. Compared with CNTR mice, CNTR+HFD mice exhibited glucose intolerance and an abnormal insulin response (Fig. 2A and B), which implied that the liver of HFD-fed mice had abnormal glucose metabolism and mild liver fibrosis had developed. Since HSC activation and ECM deposition are the main causes of fibrosis, we further detected α-SMA expression which is the marker gene of HSC activation.
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YAP signaling pathway was activated while the insulin signaling pathway was weakened in mice liver after 4 months of HFD stimulation

HFD-treated mice showed abnormal glucose metabolism, and because the insulin signaling pathway plays a key role in glucose metabolism in liver, we next tested the liver insulin signaling. After 4 months of HFD treatment, the protein expression of IRS1 did not change significantly, but the expression of IRS2 was significantly reduced. In addition, their downstream signaling molecule Akt showed a slightly increasing trend, and the ratio of p-Akt/Akt just exhibited decreased trend (p-Akt was almost undetectable when the mice were at fasted state). However, the ratio of p-FoxO1/FoxO1 was dramatically reduced which indicated that FoxO1 was activated (Fig. 3A). A similar result was obtained from immunohistochemistry (Fig. 3C and D) p-FoxO1 was decreased, whereas FoxO1 was translocated from cytosol to nucleus. These data showed that HFD disrupted the insulin signaling pathway and FoxO1 was dramatically activated in mice at fasted state. Moreover, the ratio of p-YAP/YAP in the liver was decreased after HFD stimulation. CTGF, the target of YAP, did not change significantly. On the contrary, the protein expression of Ankrd1, another target of YAP, was significantly increased (Fig. 3B) indicated that the YAP signaling pathway was activated after HFD stimulation for 4 months.

Liver FoxO1 expression was downregulated after YAP knockout in mouse liver

HFD treatment impaired the insulin signaling pathway and FoxO1 was activated in the liver. At the same time, the YAP signaling pathway was activated. Subsequently, we wanted to elucidate whether there was a cross-link between YAP and FoxO1. In order to address this question, we next selected liver-specific knockout Yap mice (L-YKO) and control mice (YapLoxp/Loxp) to detect FoxO1 expression in the liver. After Yap-specific knockout in the liver, the protein expressions of its target genes, both CTGF and Ankrd1, were all significantly reduced (Fig. 4A and B); FoxO1 was also significantly reduced via both Western blot and immunohistochemistry (Fig. 4A and C), indicating that its overall ability to degrade ECMs was decreased. These results suggested that after supplementation with a high-fat diet, excessive ECMs in the liver were deposited, and HSCs were activated during liver fibrosis induced by HFD.
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which suggested that knockout of the Yap in the liver could lead to downregulation of FoxO1 expression.

Specific knockout of YAP in liver can improve abnormal glucose metabolism and liver fibrosis induced by HFD

In order to verify the above hypothesis, 2-month-old CNTR mice (Yap\text{Loxp/Loxp}) and L-YKO mice were given HFD for 4 months. Compared with the CNTR group, body weight and liver weight of L-YKO mice were significantly reduced (Fig. 5A and B); ALT and AST in the serum of L-YKO mice were significantly decreased (Fig. 5C and D), which suggested that knockout of YAP in the liver improved the abnormal liver function induced by HFD. H&E staining results showed that the swelling of liver cells and the loosening of cell connections in the L-YKO group were all significantly recovered, and lipid droplets were significantly reduced (Fig. 5E). Meanwhile, Masson staining result revealed that the blue area around the portal area of the liver in the L-YKO group was reduced (Fig. 5F). These data suggested that the knockout Yap in mice liver can improve liver morphologic injury and fibrosis induced by HFD. Compared with the control mice, the expression of α-SMA in liver tissue was significantly reduced with Yap gene knockout (Fig. 5G and H), which also indicated that the knockout Yap gene could inhibit HSC activation and alleviate liver fibrosis induced by HFD.
Specific liver knockout of Yap reduces the expression of fibrosis-related genes in the fibrotic liver induced by HFD

Moreover, GTT and ITT tests showed that glucose metabolism was also improved after Yap knockdown in the liver (Fig. 6A and B). Compared with CNTR+HFD mice, the expressions of TGF-β1, α-SMA and Collagen1 in the liver of L-YKO+HFD mice were significantly reduced at the protein level (Fig. 6C). Meanwhile, MMP2 and MMP9 increased significantly (Fig. 6D). The data inferred that liver fibrosis was improved by increasing the expression of MMP2 and MMP9, reducing the expression of TGF-β1 and inhibiting the activation of HSC after Yap knockout in the liver.

The insulin signaling pathway was improved at FoxO1 level in the liver of Yap specifically knockout mice after giving HFD stimulation

Compared with CNTR+HFD mice, the protein expression of Yap in the liver of L-YKO+HFD mice was significantly reduced, and its targets CTGF and Ankrd1 all reduced significantly (Fig. 7A), indicating that Yap was knocked out in the liver. Ankrd1, a Yap target gene, was further suppressed. At the same time, compared with CNTR+HFD mice, the protein level of IRS1 in the liver of L-YKO+HFD
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mice was significantly downregulated, but the expression of IRS2 was significantly increased, the ratio of p-Akt/Akt remained unchanged, and the ratio of p-FoxO1/Akt was dramatically increased via Western blot detection (Fig. 7B). Moreover, the expression of FoxO1 in liver tissue of mice was significantly decreased, whereas the expression of p-FoxO1 was increased, and p-FoxO1, strikingly like YAP, was mainly located around the portal area (Fig. 7C and D). These data indicated that knockout Yap in the liver improved the insulin signal pathway at the FoxO1 level, but not at the Akt level, the upstream of FoxO1.

**FoxO1** gene is positively regulated by YAP in vitro

In order to determine whether FoxO1 gene is regulated by YAP or not, we isolated primary hepatocytes from normal mice, and transfected them with overexpressed YAP gene lentivirus in vitro. Compared with normal hepatocytes transfected with empty virus, the protein and gene expression level of YAP dramatically increased in hepatocytes with the overexpressed YAP gene, which indicated that the transfection was successful. Moreover, with the overexpression of YAP gene, the YAP target genes, such as Ankrd1 and CTGF, and FoxO1 and TGF-β1, gene expression were also significantly upregulated (Fig. 8A and B). The same results were reconfirmed in JS-1 cells (Fig. 8C). The data demonstrated that YAP positively regulated FoxO1 gene expression in vitro.

**Discussion**

In this study, we presented three important findings: (1) YAP signaling pathway was activated, IR was induced and liver FoxO1 was activated in mice liver after HFD stimulation; (2) specific knockout Yap gene in the liver improved abnormal glucose metabolism, liver injury and liver fibrosis induced by IR; and (3) the underlying mechanisms were related to YAP positively regulated FoxO1 gene expression.

Liver fibrosis is a result of chronic liver injury due to various factors, such as alcohol consumption, NAFLD, non-alcoholic steatohepatitis (NASH), viral hepatitis, autoimmune hepatitis, and cholestatic liver diseases (Aydin & Akcali 2018). IR and NAFLD have a close relationship such that NAFLD presents in up to two-thirds of patients with type II diabetes mellitus (T2DM) (Fruci et al. 2013). Moreover, IR and T2D shared common mechanisms in severe fibrosis in NAFLD patients (Angulo et al. 2015). The shared mechanisms of IR and NAFLD mainly include adipocytes releasing hormones, adipose tissue inflammation, increased serum-free fatty acids (FFA) and increased lipogenesis (Kralj et al. 2016). However, IR is an independent risk factor and one of the main pathogenesis of NAFLD, and because IR has occurred in the early stage of fatty liver (Åberg et al. 2018) it is also a common pathogenic factor of metabolic syndrome (Marchesini et al. 2005). Besides IR, NAFLD is closely related to obesity, diabetes and dyslipidemia, which promote each other and form...
a vicious circle during metabolic syndrome (Rhee 2019, Moylan et al. 2014). Especially, IR was induced both in hepatocytes and in peripheral tissue due to long-term high levels of FFA in blood, and IR further increased blood FFA. In the end, NAFLD and IR form a vicious circle (Kralj et al. 2016, Feng et al. 2017). Thus, the prevention of IR and reduction of lipid synthesis are essential for the prevention of NAFLD. Recently, alterations in peripheral glucose metabolism due to IR were assessed by HOMA-IR (Glucose x Insulin/22.5), whereas adipose tissue IR was estimated as (Adipo-IR = Free Fatty Acids x Insulin), which suggests that there is a close relationship between IR and liver fibrosis (Gaggini et al. 2019). NAFLD has been classified as a set of liver diseases ranging from steatosis to NASH, steatohepatitis with fibrosis, and cirrhosis (Mundi et al. 2020). While NAFLD and its triggered liver fibrosis have multiple pathogenesis, modulation of IR will be the mainstay of therapeutic approaches in NAFLD as it is the upstream pathogenesis of NAFLD (Khan et al. 2019).

Here, we fed 2-month-old mice with HFD to induce IR-type NAFLD and then developed liver fibrosis. We found that the liver insulin signaling pathway was weakened and liver FoxO1 was activated, which indicated that IR and mild liver fibrosis occurred with the abnormal glucose metabolism.

Liver fibrosis is characterized by excessive deposition of ECM. When liver injury occurs, HSCs were presented in the Disse space and were activated and differentiated into myofibroblast-like cells, obtaining contractile, pro-inflammatory and fibrotic characteristics. When injury occurs, resting HSC undergoes trans-differentiation from vitamin A storage cells to myofibroblast-like cells. Activated HSCs upregulate α-SMA, release excessive ECMs, including collagen and fibronectin, which play a dominant role in progressive liver fibrosis. Therefore, α-SMA was named as a marker gene for HSCs activation, and revealing the molecular mechanism that drives the early stages of HSC activation is the key to preventing and reversing the fibrosis response (Friedman 2008b, Zhang et al. 2018). We found that in mice stimulated by HFD for 4 months, the expression of fibrosis-related genes, such as α-SMA and TGF-β1, and the fiber network-related proteins, such as Collagen1, all increased. Moreover, MMPs, overall ability to degrade ECM was decreased. These data suggested that HFD resulted in ECM which was deposited in mice liver, HSCs were activated, and early stage of liver fibrosis has been developed by IR.

Moreover, transient accumulation of activated HSCs is necessary for effective liver regeneration. However, prolonged excessive activated HSCs accumulation causes...
presented in liver tissue which visually inferred that HSCs were activated.

YAP is a morphogenic signaling protein that is relatively inactive in healthy liver but significantly activated in HSCs during liver injury (Machado et al. 2015). As a nuclear effector of the Hippo signal pathway, YAP can promote cell proliferation and inhibit apoptosis through its transcriptional coactivator activity. In hepatic fibrosis, YAP is activated in HSCs in response to liver injury in vivo with an increase in the expression of its target genes, such as CTGF and Ankrd1, and activated YAP/TAZ can upregulate ECM deposition and tissue hardness, which subsequently promoted the fibrosis process (Herrera et al. 2018). In addition, activated YAP translocated into the nucleus and combined with TEAD1-4 transcriptional factors, which promoted CTGF transcription, and secreted phosphoprotein-1 and TGF-β (Seki & Brenner 2015, Dooley & ten Dijke 2012). YAP has been proved to be involved in liver fibrosis through TGF-β/Smad pathway, and YAP may be a potential target to reverse hepatic fibrosis (Mannaerts et al. 2015).

Interestingly, we found that with mild liver fibrosis development and IR occurrence, YAP phosphorylation was decreased and its target molecular gene, Ankrd1, has been upregulated, which indicated that the YAP signal was activated. Moreover, like fibrosis deposition, YAP expression was mainly located around the portal area, and nucleus YAP was indeed increased and translocated from the cytosol into the nucleus after the simulation of HFD, which is consistent with the finding that native YAP strongly localizes within the nuclei of reactive-appearing ductular cells that produce potent pro-fibrogenic factors through analysis of both people and mice with chronically injured fatty livers (Machado & Diehl 2014). Nevertheless, which is different from Ankrd1, is unchanged in this mice model, and the reason needs to be further addressed.

In addition, YAP is also involved in regulating energy homeostasis in some cells through glycolysis and glutaminolysis (Wang et al. 2015, Cox et al. 2016), suggesting that morphogens may modulate metabolism to induce cell fate changes to allow tissues to survive or recover. Furthermore, TGF-β1/Smad3 promotes hepatic gluconeogenesis via PP2A-AMPK-FoxO1 pathways during IR and diabetes (Yadav et al. 2017, Zhang et al. 2019). YAP regulates components of the AKT pathway (i.e. PI3K, PTEN, and AKT) (Cinar et al. 2007, Tumaneng et al. 2012, Ye et al. 2012, Lin et al. 2015). All these suggest that there is crosstalk between the Hippo and Insulin pathways in the maintenance of functional liver homeostasis.

**Figure 8**

YAP regulates FoxO1 changes in vitro. (A) The protein expression of YAP and FoxO1. (B) The changes of FoxO1 gene expression after primary hepatocytes were transfected with the plasmid overexpression of YAP gene (pLenti, pLenti-YAP; n=5, compared with pLenti, *P < 0.05, **P < 0.01). (C) Changes of HSC line JS-1 transfected with a plasmid overexpressing YAP gene (JS-1+pLenti, JS-1+YAP, n=3, compared with JS-1+pLenti, *P < 0.05, **P < 0.01, ***P < 0.001). (D) Proposed mechanisms of YAP involved in the liver fibrosis induced by IR-through activation of FoxO1 gene expression. Mice were given high-fat diet for 4 months to induce liver fibrosis. During the process of IR, insulin—IR1/IR2→Akt→FoxO1 signaling activity was inhibited, YAP→CTGF and Ankrd1 signaling activity was activated, TGF-β1→Smad2/3 signaling activity was enhanced, HSCs were activated, and more ECMs were deposited, which promoted the occurrence and development of liver fibrosis. After liver-specific knockout YAP gene in mice, liver dysfunction and fibrosis were significantly improved, HSCs activations were inhibited, ECMs deposition was alleviated, TGF-β1→Smad2/3 signaling activity was downregulated, and FoxO1 activity was decreased. Red arrows indicate HFD-induced effects; green arrows indicate liver-specific knockout YAP gene effects; blue arrows indicate uncertain effects and need to be further investigated. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0561.
We discovered that YAP signaling pathway was activated while the insulin activity was weakened, and liver FoxO1 was activated. Thus, we speculated that YAP signaling pathway may play an important role in the early stage of liver fibrosis induced by IR and wanted to detect if there is a crosslink between YAP and FoxO1 or not.

To address this question, we used the Cre/loxP genetic method to specifically knockout Yap gene in mouse liver. We found that abnormal glucose metabolism, liver damage and liver fibrosis were all improved in HFD-treated mice. FoxO1 was also significantly reduced with downregulated YAP target genes, including both CTGF and Ankrd1. The activation of HSC was inhibited as a result of decrease in α-SMA expression. The expression of fibrotic related genes, such as TGF-β1 and Collagen1, in the liver were both significantly reduced, whereas MMP2 and MMP9 increased, which showed that knockdown Yap in the liver can improve liver fibrosis induced by HFD in mice. Notably, liver insulin signal activity was also improved, and the activity of FoxO1 was downregulated and phosphorylated FoxO1 expression increased around the portal area.

In addition, in our cell-based studies, transient overexpression of YAP gene through transfection with YAP high-expression virus, FoxO1 gene expression increased, which further directly confirmed that YAP positively regulated FoxO1 gene.

Recently, YAP and TGF-β/Smad signaling pathways were activated and played important roles in the development of NAFLD and in injured livers (Chen et al. 2018, Oh et al. 2018). Here, we provided a new mechanism that YAP knockout can improve mild liver fibrosis induced by IR through positively regulating FoxO1, a key downstream molecular gene in the insulin signaling pathway. However, the mutual regulation of YAP by FoxO1 or how FoxO1 regulates YAP needs to be studied further. Anyway, YAP and FoxO1 were activated and played a key role in the occurrence and development of liver fibrosis induced by IR, and YAP positively regulated FoxO1 in the liver to improve liver fibrosis has become a new mechanism.

YAP and TGF-β signaling pathways and the interactions between them promote the development of NAFLD (Chen et al. 2018), while TGF-β not only regulates transcription through Smad signal pathway, but also consists of a series of secretory factors (Narimatsu et al. 2015, Glrizes et al. 1997, Meindl-Beinker & Dooley 2008). Moreover, the activation of HSCs involves a series of signal transduction mechanisms, including Wnt, notch, hippo and TGF-β/Smad pathway (Geisler & Strazzabosco 2015). Each pathway interfaced jointly into a network to regulate the occurrence of liver fibrosis. Among them, Yap/TAZ, as the main downstream molecule of the Hippo pathway, holds a key position in liver fibrosis. Yap/TAZ also reacts with Smad, Runx1/2, p73, ErbB4, Pax3 and TBX5 transcription factors which regulate the expression of specific genes (Wang et al. 2009). Since YAP positively regulated FoxO1 gene, whether FoxO1 also regulates TGF-β/Smad pathway in the development of NAFLD and fibrosis induced by IR, also needs to be further investigated.

Additionally, in order to avoid bias in the analysis of non-differentiated HSC resulting in ECM deposition and the role of YAP and FoxO1, we also detected α-SMA expression to visually observe HSCs activation and YAP, p-FoxO1 and FoxO1 expression in the liver using immunohistochemistry. Plus that the result protein expression via Western blot the analysis, and the fibrosis deposition via Masson staining, we believed that the bias will be at least partly avoided. However, the question needs to be fully explored more directly regarding the process of HSC differentiation in future studies.

To sum up, in spite of the complicated factors involved in liver fibrosis, our study revealed that YAP plays a key role in the occurrence and development of steatosis in the early stage of fibrosis induced by IR. Liver-specific knockdown YAP gene can improve liver injury and liver fibrosis induced by IR, and the study of these mechanisms may be related to the positive regulation of FoxO1 and improved insulin activity at the FoxO1 level. These findings shed new light on the study of liver fibrosis induced by IR, and YAP is expected to be a new therapeutic target.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Yujiao Dai, Peng Hao and Zhimei Sun researched data and wrote the manuscript; Hongyu Song, Yida Li researched data; Lihui Xue and Mingming Gao prepared liver protein and RNA; Zhiyi Guo and Hong Xu revised the article and improved related experiments; Shuang Li and Teng Si maintained mice; Yuxin Zhang researched data; Yajuan Qi designed, supervised the research and wrote the manuscript.

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