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Mst1 regulates hepatic lipid metabolism by inhibiting Sirt1 ubiquitination in mice

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ABSTRACT

Previous study showed mammalian Ste20-like kinase (Mst1) may serve as target for the development of new therapies for diabetes. However, the function of Mst1 involved in liver lipid metabolism has remained elusive. In this study, we report that the liver of *Mst1* knockout (*Mst1*$^{-/-}$) mice showed more severe liver metabolic damage under fasting and high-fat diet than that of control mice. And fasting induced hepatic *Mst1* expression. Mst1 overexpression inhibited *Srebp-1c* expression and increased the expression of antioxidant genes in primary hepatocytes. We also found that fasting-induced expression of hepatic Sirt1 was attenuated in *Mst1*$^{-/-}$ mice. Mst1 overexpression promoted Sirt1 expression, probably due to inhibiting Sirt1 ubiquitination. In summary, our study suggests that Mst1 regulates hepatic lipid metabolism by inhibiting Sirt1 ubiquitination in mice.

1. Introduction

With the increase in the global obesity population, the incidence of non-alcoholic fatty liver disease (NAFLD) is expected to increase. NAFLD is defined as fat accumulation in greater than 5% of liver cells known as hepatocytes [1]. NAFLD represents a spectrum of hepatic pathologies, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis [2]. Individuals with NASH are at increased risk of developing cirrhosis, hepatic decompensation, and hepatocellular carcinoma [3]. The molecular pathogenesis of NAFLD is complex and involves multiple genetic and environmental factors. Some studies have indicated that the pathogenesis of NAFLD is characterized by the 'two hit' model [4]. The first hit occurs when insulin resistance causes lipid accumulation in hepatocytes, and the second hit occurs when cellular insults, resulting in inflammation and fibrosis from oxidative stress, mitochondrial dysfunction, lipid peroxidation and cytokine activity [5]. However, there is increasing evidence that free fatty acids can directly cause toxicity by increasing oxidative stress and by activation of inflammatory pathways, therefore hepatic triglyceride accumulation may be a protective mechanism by preventing the toxic effects of unesterified FFA [6]. Additionally, the development of fibrosis/cirrhosis is dependent on the efficacy of hepatocyte regeneration and oxidative stress inhibits the replication of mature hepatocytes which replace the dead cells and reconstitute normal tissue function, therefore cell death with impaired proliferation of hepatocyte progenitors represents the proposed'third hit'in NAFLD pathogenesis [6].

The mammalian Ste20-like kinases (Msts), of which Mst1 and Mst2 share the highest degree of homology and are ubiquitously expressed serine–threonine kinases. *Mst1* is part of the Hippo signaling pathway and involved in multiple cellular processes such as morphogenesis, proliferation, stress response and apoptosis [7,8]. Mst1 can phosphorylate the FoxO transcription factors at a site that is conserved within the forkhead domain of these proteins from mammals to *C. elegans*. Sirt1 is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase and serves as an energy sensor to modulate insulin sensitivity through its substrates,

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most importantly FoxO1 [9]. Previous study demonstrated that Mst2 and Salvador homolog 1 (Sav1), a scaffolding protein that functions in the *Mst* pathway, significantly increased *PPARγ* transactivation and augmented differentiation of 3T3-L1 cells, indicating *PPARγ* activation by the *Mst* signaling pathway may be a novel regulatory mechanism of adipogenesis [10]. Recently, Ardestani et al. reported Mst1 as a proapoptotic kinase and key mediator of apoptotic signaling and beta cell dysfunction and suggest that Mst1 may serve as target for the development of new therapies for diabetes [11]. However, the function of Mst1 involved in liver lipid metabolism has remained elusive.

In this study, we report that HFD aggravated liver metabolic damage in *Mst1*$^{-/-}$ mice. In primary hepatocytes, MST1 overexpression promoted Sirt1 expression by inhibiting Sirt1 ubiquitination, furthermore decreased *Srebp-1c* expression and improved antioxidant genes expression. Our results suggest that *Sirt1* mediates the effect of *Mst1* on hepatic lipid metabolism.

2. Materials and methods

2.1. Animal protocols

Mst1-deficient (*Mst1*$^{-/-}$) mice and wild-type 129 mice were provided by Professor Tao (Fudan University, China). Generation of *Mst1*$^{-/-}$ mice was previously reported [12]. All mice were housed and maintained on a 12 h light–dark cycle and a regular unrestricted diet. The mice were fed with either a normal chow (9% fat; Lab Diets) or HFD (45% fat; Research Diets) and libitum with free access to water. The diagnosis of nonalcoholic steatohepatitis (NASH) is defined by the presence and pattern of specific histological abnormalities on liver biopsy according to NAFLD Activity Score (NAS) [13]. All animal experiments were conducted under protocols approved by the Animal Research Committee of the Institute of Laboratory Animals, Chinese Academy of Medical Sciences and Peking Union Medical College. All the surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Histological analysis

For H&E staining, liver tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 7-μm sections. For Oil Red O staining, liver tissue was frozen in liquid nitrogen and cut into 10-μm sections. Sections were stained and analyzed at 20 × magnification using a microscope.

2.3. Cell culture

Primary hepatocytes were isolated and cultured as previously reported [14]. Mouse hepatocytes were infected by adenovirus expressing His-tagged Mst1. 293A cells were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% (v/v) penicillin-streptomycin. Cell transfection was performed according to the manufacturer's protocol (Vigorous).

2.4. Western blotting

Protein was extracted from frozen liver samples or cultured hepatocytes in cell lysis buffer. In total, 40–60 μg of protein was loaded onto a 10% SDS-polyacrylamide gel, and separated proteins were transferred to PVDF membranes. Western blot assays were performed using specific antibodies. Anti-Sirt1 antibody was purchased from Cell Signaling Technologies (Beverly, MA). Anti-Ho-1 and -Sod2 antibody were from ABclonal Technology. Anti-α-tubulin was obtained from Abmart (Arlington, MA).

2.5. RNA isolation and quantitative RT-PCR

Total RNA was extracted from the primary hepatocytes using a TRIzol-based method (Roche). Approximately 2 μg of total RNA was reverse-transcribed into a first-strand cDNA pool using Super-Script™ reverse transcriptase and random primers (Abcam). Quantitative real-time reverse-transcriptase PCR was performed using the SYBR Green I Q-PCR kit (Promega) on a Bio-Rad CFX system. All gene expression data were normalized to β-actin expression levels. Primer sequences are available upon request.

2.6. Statistical analysis

All the data are indicated as mean ± s.d. Differences among means are analyzed by Independent-sample T test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Fasting and high-fat diet aggravate liver metabolic damage in *Mst1*$^{-/-}$ mice

To study the function of *Mst1* gene in liver metabolism, we first analyzed the liver phenotypic response of *Mst1*$^{-/-}$ mice to fasting. We found that fasting increased hepatic lipid accumulation and showed hepatic steatosis in the control mice (Fig. 1A), however, the liver damage was aggravated in *Mst1*$^{-/-}$ mice, displaying accumulations of lipid droplets and degeneration of ballooning in liver (Fig. 1A). Then, we evaluated the liver damage when *Mst1*$^{-/-}$ mice were fed by HFD for 24w. The results showed that *Mst1*$^{-/-}$ mice on HFD displayed much worse phenotypes while 129 mice on HFD presented with apparent hepatosteatosis, including massive accumulations of large lipid droplets and ballooning degeneration of liver cells (Fig. 1B).

3.2. Mst1 decreases Srebp-1c expression and increases the expression of antioxidant genes

Though fasting induces the change of activation and expression of many genes, we examined the effect of fasting on *Mst1* expression. The results showed that fasting induced the mRNA expression of *Mst1* in mouse livers (Fig. 2A). Lipid accumulation in the liver is a major hallmark of NAFLD and *Srebp-1c*, a key transcription factors regulating hepatic lipid metabolism, has been proposed to have great potential for NAFLD treatment [15]. Collective studies have suggested that oxidative stress may also contribute to clinical progression from simple fatty liver to NASH [16]. So, we studied the impact of *Mst1* on *Srebp-1c* and some antioxidant genes. Our results showed that *Mst1* overexpression decreased the mRNA level of *Srebp-1c*, and increased the mRNA level *FoxO1*, *Ho-1*, *Sod2*, *Tfam* and *Gpx1* in mouse primary hepatocytes infected with Ade-*Mst1* (Fig. 2B). In addition, we found that *Mst1* overexpression increased the protein expression level of Ho-1 and Sod2 (Fig. 2C) and reduced mitochondrial ROS production in WT primary hepatocytes (Fig. 2D).

3.3. Fasting-induced hepatic Sirt1 increase is attenuated in *Mst1*$^{-/-}$ mice

Previous studies have indicated that fasting induced the expression of *Sirt1* and activation of *Sirt1* ameliorated hepatic steatosis by inhibiting *Srebp-1c* expression and increasing antioxidant capacity in the liver [17–21], thus we examined whether the

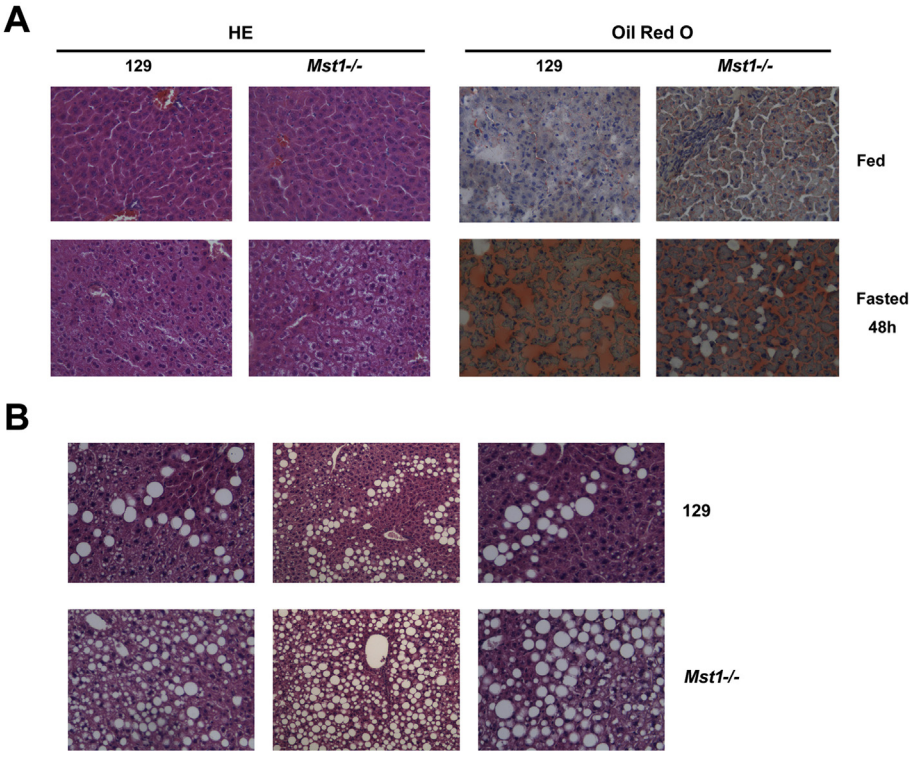

Fig. 1. Fasting and High-fat diet aggravated liver metabolic damage in *Mst1*$^{-/-}$ mice. (A) H&E and Oil Red O staining from 129 mice and *Mst1*$^{-/-}$ mice on a standard diet or fasted for 48 h. (B) H&E staining from 129 mice on high-fat diet (HFD) and *Mst1*$^{-/-}$ mice on HFD for 24w.

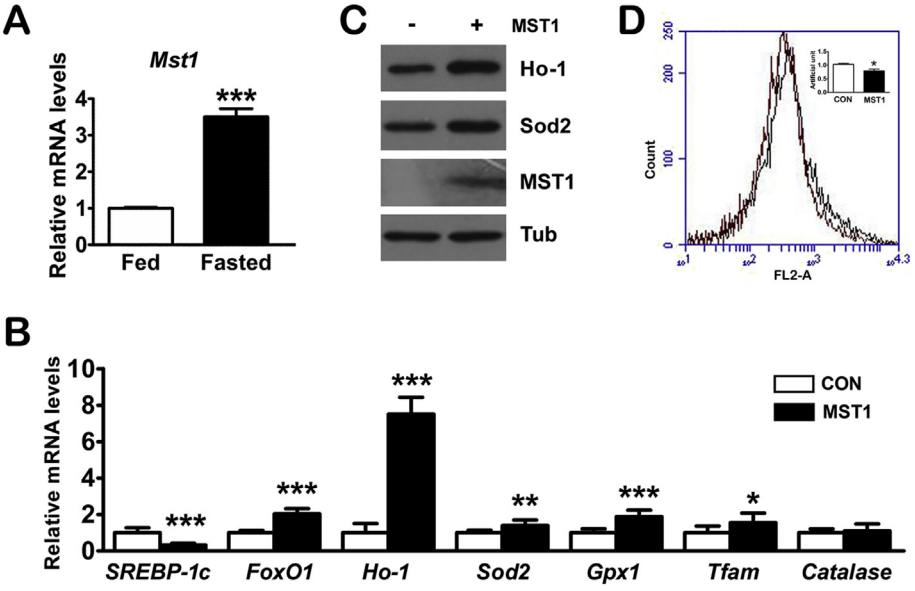

Fig. 2. Mst1 decreases Srebp-1c expression and increases the expression of antioxidant genes. (A) Fasting induces hepatic mRNA expression of Mst1. 129 mice were fed on a standard diet or fasted for 24 h. (B) The effect of Mst1 on the mRNA levels of Srebp-1c and antioxidant genes in primary hepatocytes infected with Ade-Mst1. (C) MST1 increases the protein levels of Ho-1 and Sod2 in primary hepatocytes. (D) MST1 inhibits ROS production in primary hepatocytes.

Sirt1 expression of induced by fasting was affected in *Mst1*$^{-/-}$ mice. As previous reported, our result showed that fasting induced hepatic Sirt1 expression in 129 mice (Fig. 3). However, the effect of fasting-induced hepatic Sirt1 increase was attenuated in *Mst1*$^{-/-}$ mice (Fig. 3). These results indicate that Mst1 may involve in fasting-induced *Sirt1* expression.

3.4. *Mst1 increases the expression of Sirt1 by inhibiting Sirt1 ubiquitination*

To further explore the effect of *Mst1* on Sirt1, we performed the following experiments. The result showed that *Mst1* overexpression enhanced Sirt1 expression in primary hepatocytes (Fig. 4A) and 293A cells (Fig. 4B). However, Mst1^{K59R}, a kinase-dead

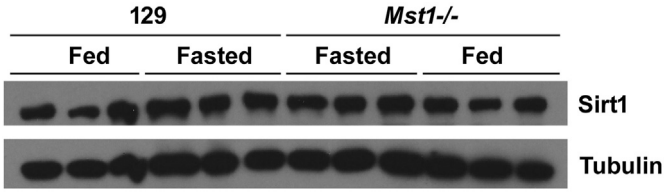

Fig. 3. Fasting-induced hepatic Sirt1 increase is attenuated in Mst1$^{-/-}$ mice. The 8-10 week-old mice were fed with a standard diet or were fasted for 24 h, then the liver were dissected and the proteins were isolated, the protein levels of Sirt1 and α-tubulin were tested by western blotting.

study, we found that both of fasting and high-fat diet cause fat accumulation in the liver of 129 mice, and this phenomenon was aggravated in the liver of *Mst1*$^{-/-}$ mice. In addition, *Mst1*$^{-/-}$ mice also displayed hepatocyte swelling or ballooning degeneration and lobular inflammation in the liver. It is now well known that fasting causes a rapid transcriptional activation of genes encoding mitochondrial, peroxisomal and microsomal fatty acid oxidation in liver in healthy individuals [25,26]. Mst1 activity was highly upregulated upon chronic exposure to increasing glucose concentrations or palmitic acid, or upon exposure to acute oxidative stress from hydrogen peroxide [11]. In this study, we show that fasting induces

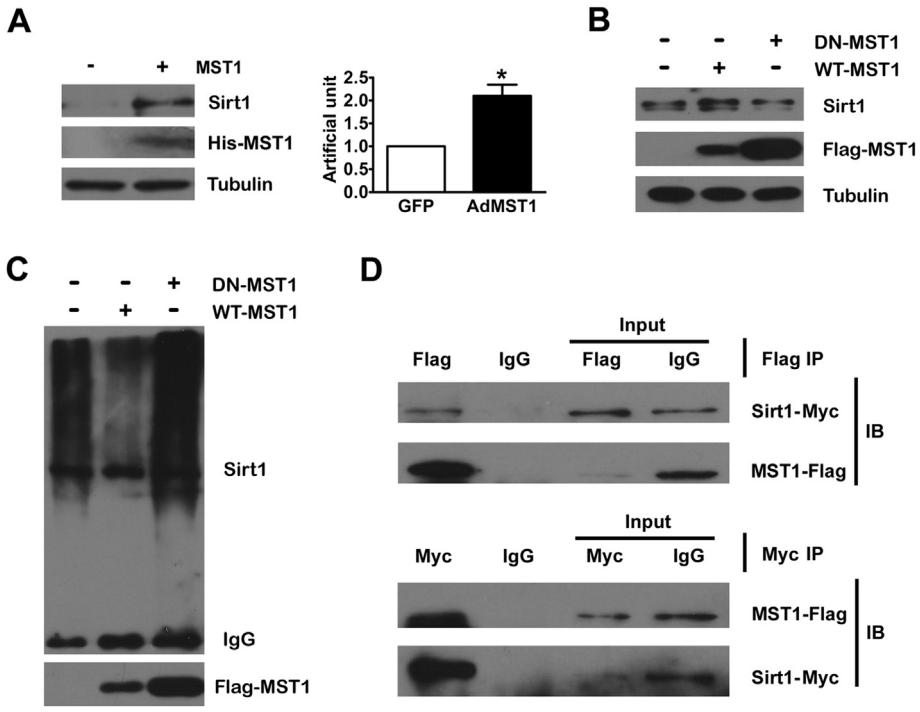

Fig. 4. Mst1 increases the expression of Sirt1 by inhibiting Sirt1 ubiquitination. Mst1 induces Sirt1 expression in primary hepatocytes (A) and 293A cells (B). (C) Mst1 inhibits Sirt1 ubiquitination. Immunoblotting with HA antibody after immunoprecipitation with an anti-Myc antibody of 293A cells transfected with Myc-Sirt1 and HA-ubiquitin (Ub), alone or together with Flag-MST1 (WT-MST1) or Flag- Mst1^{K59R} (DN-MST1) expression plasmids for 48 h (D) MST1 interacts with Sirt1. 293A cells were transfected using the expression plasmids of Flag-MST1 and Myc-Sirt1. After 48 h transfection, the proteins were isolated and the interaction of MST1 and Sirt1 was tested by co-immunoprecipitation.

Mst1 in which the ATP binding site was mutated, could not increase the expression of Sirt1 in 293A cells (Fig. 4B). Furthermore, we found overexpression of Mst1, but not Mst1^{K59R}, inhibited Sirt1 ubiquitination in 293A cells (Fig. 4C). Moreover, our study revealed that the Mst1 protein was co-immunoprecipitated with Sirt1, further confirming interaction between Mst1 and Sirt1 proteins (Fig. 4D).

4. Discussion

Previous studies have indicated Mst1 as a proapoptotic kinase and key mediator of apoptotic signaling and beta cell dysfunction and suggested that it may serve as a target for the development of new therapies for diabetes [11]. However, the role of *Mst1* gene in liver metabolism remains unclear. The minimal histologic criteria for the diagnosis of NASH include fat accumulation and the presence of hepatocyte swelling or ballooning degeneration and lobular inflammation [22]. A separate histologic scoring system for the spectrum of NAFLD, called the NAFLD Activity Score (NAS), was developed as a tool to measure changes in NAFLD [13,23,24]. In this

the expression of *Mst1*.

Srebp-1c has been identified as a key transcriptional regulator involved in liver lipid homeostasis [27]. Our result demonstrated that *Mst1* overexpression inhibited *Srebp-1c* expression in primary hepatocytes. Srebp-1c levels were elevated in the fatty livers of different animal models including obese, insulin-resistant and hyperinsulinaemic ob/ob mice and were also depressed during fasting but increased markedly when animals were refed a high carbohydrate diet [28]. Collective studies have indicated that oxidative stress and mitochondrial dysfunction play an important role in the pathogenesis of NAFLD [6]. Oxidative stress induces Srebp-1c activation and liver steatosis, the mechanisms of this process likely include an activation of the endoplasmic reticulum stress pathway [28]. In addition, β-oxidation taking place in the mitochondria can become overwhelmed in NAFLD and give rise to excessive ROS. Excessive ROS induce oxidative stress, activate inflammatory pathways and damage mitochondrial function, eventually lead to steatohepatitis and/or fibrosis [6]. So, antioxidant strategy as a new therapeutic direction may prevent the evolution of metabolic diseases, such as NAFLD and type 2 diabetes. In this

study, *Mst1* overexpression increased the expression antioxidant genes, furthermore decreased ROS production. Previous study indicated that *Mst1* has an important role in diverse biological processes including cellular responses to oxidative stress and longevity [29]. In addition, peripheral T cells from both *Mst1*-deficient human and mice display decreased FoxO protein levels [12,30,31]. Consistently, the FoxO targets, Sod2 and catalase, were significantly down-regulated in $MST1^{-/-}$ T cells, thereby resulting in elevated levels of ROS and induction of apoptosis [30]. Recently, Li et al. demonstrated that *Mst1* differentially regulated TLR3/4/9-mediated inflammatory responses in macrophages and thereby is protective against chronic inflammation-associated hepatocellular carcinoma [32]. It is well known that the presence of steatosis is tightly associated with chronic hepatic inflammation, an effect in part mediated by activation of the Ikk-β/NF-kB signaling pathway [6]. And the chronic inflammatory state associated with hepatic steatosis may also play a key role in hepatocellular carcinoma development.

Sirt1 has emerged as a key metabolic sensor in various metabolic tissues. In response to different environmental stimuli, Sirt1 directly links the cellular metabolic status to the chromatin structure and the regulation of gene expression, thereby modulating a variety of cellular processes such as energy metabolism and stress response [33]. In addition, calorie restriction can increase the NAD^+/NADH ratio in cells and result in activation of Sirt1, furthermore extends life spans in a wide variety of species [34]. Previous study also showed that Sirt1 protein levels were induced after fasting and returned to nearly control levels upon refeeding [21]. In this study, the effect of fasting-induced hepatic Sirt1 increase was attenuated in $Mst1^{-/-}$ mice. Above these results indicate *Mst1* involved in fasting-induced Sirt1 expression in the liver. Furthermore, our results showed that *Mst1* overexpression enhanced Sirt1 expression, probably due to Mst1-mediated inhibition of Sirt1 ubiquitination. Previous studies have demonstrated that Mst1 involved in some proteins ubiquitination [11,35]. In addition, Yuan et al. reported that Mst1 can phosphorylate Sirt1 in vitro and in vivo [36]. Reversible posttranslational modification is a versatile way to regulate protein activity and crosstalk between different types of posttranslational modification has become prominent more in eukaryotic biology. Particularly prominent are the multiple connections between phosphorylation and ubiquitination, which act either positively or negatively in both directions to regulate these processes [37]. Previous studies have indicated that Sirt1 can ameliorate hepatic steatosis by inhibiting *Srebp-1*c expression and increasing antioxidant capacity in the liver [17–20]. In this study, we report that *Mst1* involved in hepatic lipid metabolism by decreasing *Srebp-1c* expression and increasing antioxidant genes expression, which may be mediated by Sirt1.

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Transparency document

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