

# Pivotal Role of TNF- $\alpha$ in the Development and Progression of Nonalcoholic Fatty Liver Disease in a Murine Model

## Authors

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## ABSTRACT

Previously, we have shown that the adipocyte-specific nuclear form of sterol regulatory element-binding protein-1c (nSREBP-1c) transgenic mice spontaneously developed hepatic lesions that are similar to those of human nonalcoholic steatohepatitis (NASH) with a concomitant elevation of plasma TNF- $\alpha$ . In this study, we analyzed the role of TNF- $\alpha$  in the progression of nonalcoholic fatty liver disease (NAFLD). We established a Tnf knockout nSREBP-1c transgenic mouse line. Glucose tolerance and liver histology were examined at the age of 20 weeks. The gene expression and protein levels were assessed by quantitative RT-PCR and Western blot, respectively. The Tnf knockout improved glucose tolerance and significantly reduced the prevalence of hepatic steatosis (20% vs. 100%,  $p < 0.0001$ ) and fibrosis (15% vs. 65%,  $p = 0.0057$ ). The expressions of Aca, Scd1, Mcp1, Tgfb1, Col1a1, and Timp1 were increased in the liver from the original nSREBP-1c transgenic mice. However, gene upregulation was reduced in the livers from the Tnf(-/-) nSREBP-1c transgenic mice. Furthermore, the hepatic levels of TIMP1 protein were increased in the original nSREBP-1c transgenic mice but not in Tnf(-/-) nSREBP-1c transgenic mice. To assess the direct effect of TNF- $\alpha$  on the expression of the genes, we cultured primary hepatocytes in the presence of TNF- $\alpha$  and found that TNF- $\alpha$  increased the expression of Mcp1, Tgfb1, and Timp1 in hepatocytes. These observations indicate that TNF- $\alpha$  plays a pivotal role in the development of NAFLD and progression to NASH through upregulating key molecules associated with lipid metabolism, inflammatory cytokines, and fibrosis in the liver.

## Introduction

Nonalcoholic fatty liver disease (NAFLD) is a potentially progressive disorder encompassing simple steatosis, nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [1–3]. The prevalence of NAFLD is increasing worldwide [4–7], and it is becoming the predominant cause of chronic liver disease. Although the pathogenesis of NAFLD is apparently associated with obesity, insulin resistance, and type 2 diabetes, the mediators of the progression are incompletely understood.

Previously, we reported that the adipocyte-specific nuclear form of sterol regulatory element-binding protein-1c (nSREBP-1c) transgenic (Tg) mice [8], which showed lipodystrophy and insulin resist-

ance, spontaneously developed hepatic lesions that are similar to those of human NASH with age [9]. In nSREBP-1c Tg mice, circulating levels of TNF- $\alpha$  and adiponectin are significantly higher and lower, respectively, than in wild-type mice [9]. TNF- $\alpha$  is one of the major proinflammatory cytokines involved in the pathophysiology of many inflammatory disorders, including low-grade chronic inflammation associated with metabolic syndrome. TNF- $\alpha$  also induces insulin resistance by promoting the serine-phosphorylation of insulin receptor substrate-1 (IRS-1) [10]. In contrast, adiponectin exerts anti-inflammatory actions [11] and enhances insulin sensitivity, counteracting the effect of TNF- $\alpha$  [12]. Thus, the imbalance of adipocytokines, which is characteristic of metabolic syndrome,

► **Table 1** Primer sequences.

Gene		Primer	Product (bp)
Acaca	Forward	5'-ATGGCGTCCGCTCTGTGATAG	130
	Reverse	5'-TGCTGGGTCATGTGGACGA	
Scd1	Forward	5'-TTCTTGGGATACACTCTGGTGC	98
	Reverse	5'-CGGGATTGAATGTCTTGTCGT	
Mcp1	Forward	5'-CTTCTGGGCTGCTGTTC	127
	Reverse	5'-CCAGCCTACTCATTGGGATCA	
Tgfb1	Forward	5'-CGTCAGACATTCGGGAAGC	179
	Reverse	5'-CAGCCACTCAGCGTATCA	
Timp1	Forward	5'-TGGGAAATGCCGCAGATATC	66
	Reverse	5'-TGGGACTTGTGGGCATATCC	
Col1a1	Forward	5'-GAGCGGAGAGTACTGGATCG	158
	Reverse	5'-TGGGACTTGTGGGCATATCC	

is likely involved in the development of steatohepatitis. We have previously reported that the transgenic expression of full length adiponectin successfully ameliorated hepatic lesions in nSREBP-1c Tg mice [13]. In this study, we assessed the role of TNF- $\alpha$  in the progression of NAFLD using TNF- $\alpha$  deficient nSREBP-1c Tg mice.

## Materials and Methods

### Animals

Male transgenic mice (C57BL/6 background) expressing nSREBP-1c in adipose tissue and TNF- $\alpha$  knockout [Tnf(-/-)] mice were purchased from Jackson, Bar Harbor, ME, and they were bred in our laboratory where they mated with wild-type C57BL/6 mice (Nippon CLEA, Shizuoka, Japan). We established a Tnf(-/-) nSREBP-1c Tg murine line on a C57BL/6 background. All mice were allowed free access to standard mouse chow pellets (347 kcal/100 g, protein 24.9 g/100 g, fat 4.6 g/100 g, Nippon CLEA) and tap water ad libitum. Mice were sacrificed at 20 weeks of age and used for study. All mice were treated in accordance with the guidelines for the care and use of laboratory animals of Kurume University School of Medicine, based on the National Institutes of Health Guidelines. Mice were sacrificed under anesthesia, and all efforts were made to minimize suffering.

### Histological studies

Liver tissues were fixed in neutral formalin and embedded in paraffin, and the sections were treated with hematoxylin and eosin staining for standard microscopy or Azan-Mallory staining to observe the location of the extracellular matrix. Hepatic steatosis was determined when the extent of fatty infiltration was > 33%.

### Western blot analysis

Liver tissue samples were lysed in ice-cold lysis buffer containing 1 mmol/l dithiothreitol, 0.0025% NP40 and a cocktail of proteinase inhibitors. The lysates were centrifuged at 19 000 g and 4 °C for 15 min, and the supernatants were collected as whole-cell extracts.

The total protein concentration of the whole-cell mixture was measured using the Bradford reagent (Bio Rad, Hercules, CA, USA). After heating at 100 °C for 5 min, 20  $\mu$ g of total protein was loaded into each well and then separated by 7.5% or 12.5% SDS-PAGE (Wako, Osaka, Japan) before transfer to nitrocellulose membranes. The membranes were incubated with 1:3200 rabbit polyclonal antibodies against mouse nuclear factor-kappa B p65 (NF- $\kappa$ B) (Abcam, Cambridge, UK) or goat polyclonal antibodies against mouse tissue inhibitor of metalloproteinases 1 (TIMP1) (R & D systems, Minneapolis, MN, USA) at room temperature for 1 h. After washing, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (Wako) or peroxidase-conjugated rabbit anti-goat IgG (Novus, Littleton, CO, USA) and then visualized using an ECL system (GE Healthcare, Buckinghamshire, UK).

### Immunohistochemical staining

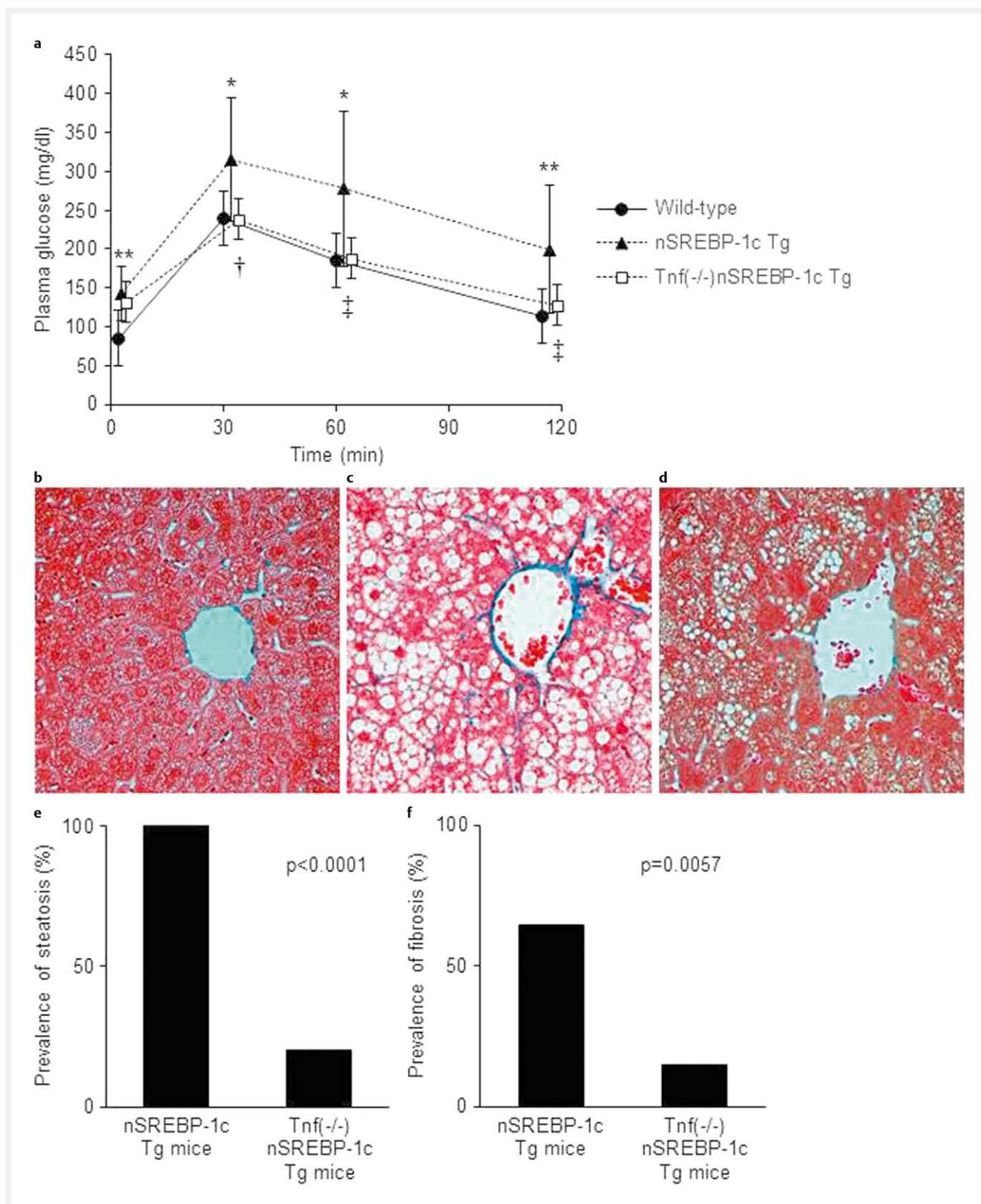
Liver tissues were embedded in paraffin and sectioned at 3  $\mu$ m thickness. After incubation in 3% H<sub>2</sub>O<sub>2</sub>, sections were blocked with 10% donkey serum albumin. Immunohistochemical staining was performed using goat anti-TIMP1 antibody (R & D systems, Minneapolis, MN, USA) and Alexa Fluor 488-labeled donkey anti-goat IgG (H + L) (Life Technologies, Carlsbad, CA, USA).

### Quantitative real-time RT-PCR

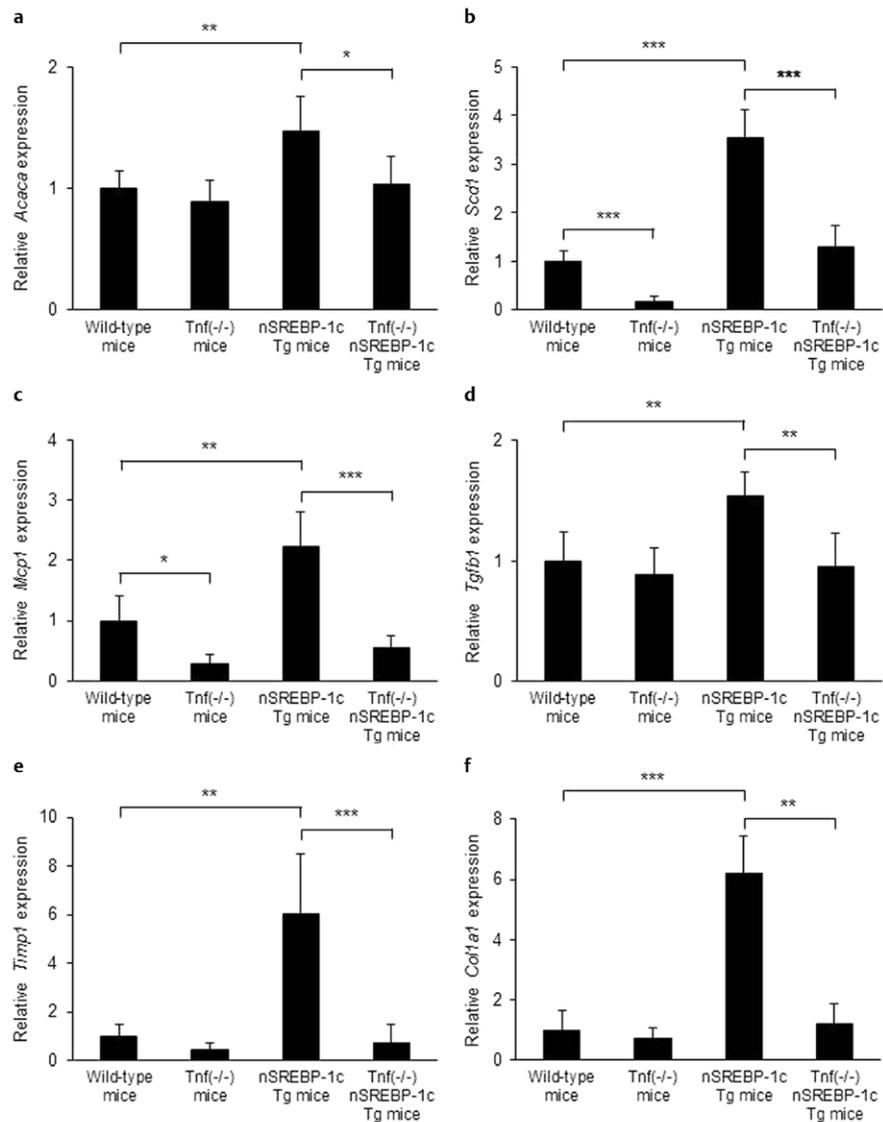
RNA was isolated from liver tissues using the RNA-Bee reagent (TEL-TEST, Friendswood, TX, USA) according to the manufacturer's instructions. The RNA concentrations were assessed by measuring the absorbance at 260 nm. For reverse transcription, 5  $\mu$ g of total RNA was transcribed to cDNA using a SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The mRNA levels of acetyl-CoA carboxylase-1 (Acaca), stearoyl-CoA desaturase-1 (Scd1), monocyte chemotactic protein-1 (Mcp1), transforming growth factor- $\beta$  (Tgfb1), tissue inhibitor of metalloproteinase 1 (Timp1), and collagen type I  $\alpha$  1 (Col1a1) genes were determined by quantitative real-time RT-PCR using StepOnePlus (Applied Biosystems, Tokyo, Japan). Primer sequences are shown in ► **Table 1**. The PCR cycling conditions were 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 60–64 °C, and 30 s at 72 °C. The relative expression was calculated as the expression of the target gene divided by the expression of the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) gene.

### The isolation and culture of primary hepatocytes

Primary hepatocytes were isolated from Tnf(-/-) C57BL/6 mice that were aged 12 weeks using a two-step collagenase perfusion method [14] with minor modifications. Briefly, the liver was perfused with Hank's balanced salt solution containing 0.5 mmol/l EGTA and thereafter with 0.05% collagenase (Nitta Gelatin Inc., Osaka, Japan). The liver was then removed and minced in Ca-free Hank's balanced salt solution, then filtered and centrifuged at 50 g and 4 °C for 1 min. Cell sediments were re-suspended with Williams' medium E supplemented with 10% fetal calf serum (Sigma, USA), antibiotics, insulin, and dexamethasone. After overnight culture in 95% air and 5% CO<sub>2</sub> at 37 °C, cells were exposed to recombinant murine TNF- $\alpha$  (20 ng/ml, Wako, Tokyo, Japan). The mRNA levels of Acaca, Scd1, Mcp1, Tgfb1, Timp1, and Col1a1 were determined by quantitative real-time RT-PCR.



**► Fig. 1** Intraperitoneal glucose tolerance tests (1 g/kg body weight) in wild-type C57BL/6 mice (closed circle, n = 11), nSREBP-1c transgenic mice (closed triangle, n = 10), and Tnf(-/-) nSREBP-1c transgenic mice (open square, n = 18) at the age of 20 weeks **a**. Mean and SD. \* p < 0.05 and \*\* p < 0.01 vs. wild-type mice; † p < 0.05 and ‡ p < 0.01 vs. nSREBP-1c Tg mice. Azan-Mallory staining of liver sections from wild-type **b**, nSREBP-1c transgenic **c**, and Tnf(-/-) nSREBP-1c transgenic mice **d** at the age of 20 weeks. The prevalence of liver steatosis **e** and fibrosis **f** in liver from 20-week-old nSREBP-1c transgenic mice (n = 17) and age-matched Tnf(-/-) nSREBP-1c transgenic mice (n = 20).



► **Fig. 2** The expression of *Acaca* **a**, *Scd1* **b**, *Mcp1* **c**, *Tgfb1* **d**, *Timp1* **e**, and *Col1a1* **f** in liver from wild-type mice, *Tnf(-/-)* mice, nSREBP-1c Tg mice, and *Tnf(-/-)* nSREBP-1c Tg mice at 20 weeks of age. Mean and SD, n=4-6. \* p<0.05, \*\* p<0.01, \*\*\* p<0.0001.

## Statistical analysis

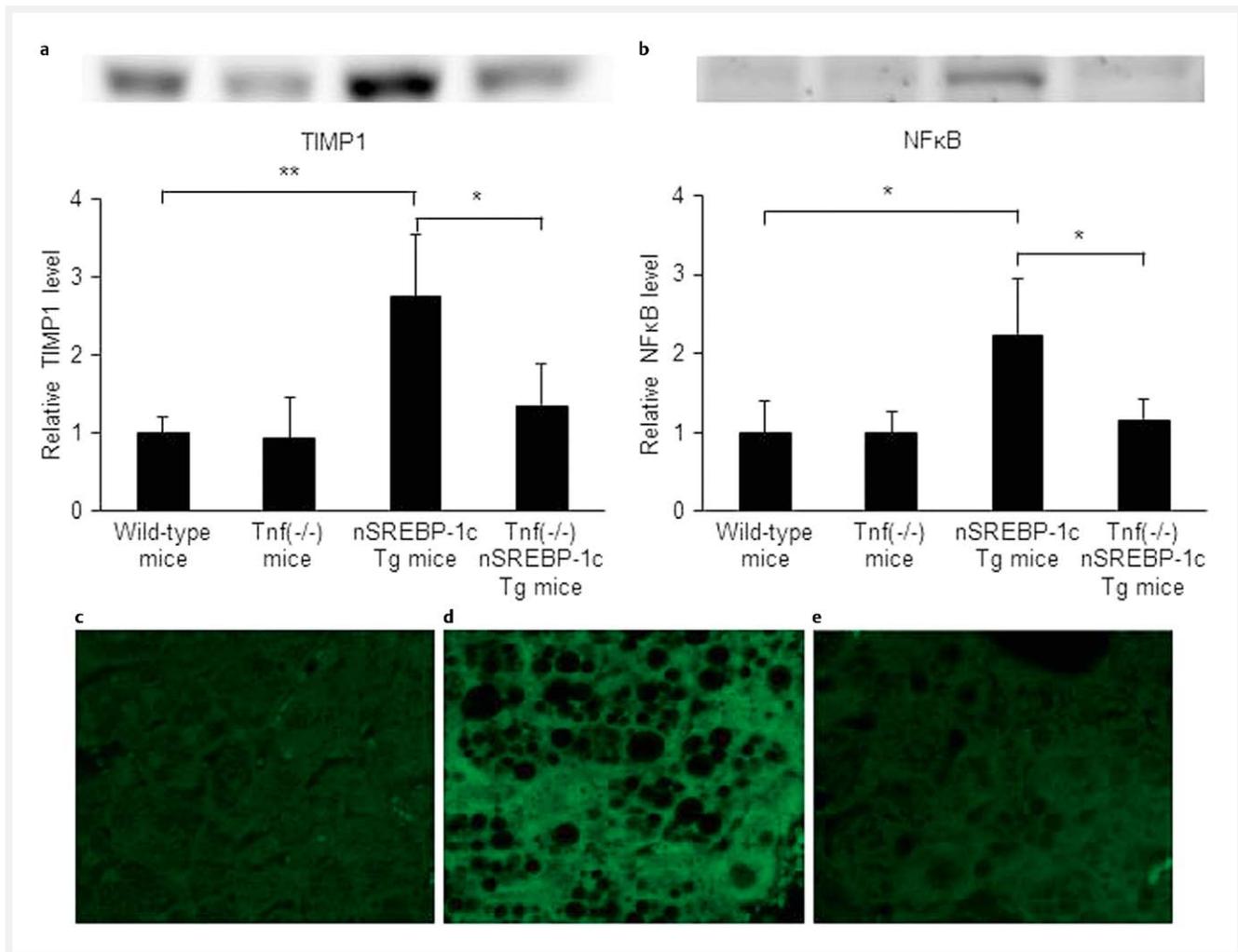
Data are expressed as the means and SD. Statistical analysis was performed using SAS v.9.3 (SAS Institute, Cary, NC, USA). ANOVA and Student's t-test were used to compare the differences between groups. The Chi-square test were used with Yates correction to compare the prevalence. The results with a p<0.05 were considered statistically significant.

## Results

### Phenotypes of TNF- $\alpha$ deficient nSREBP-1c Tg mice

There were no significant differences in the body weight among wild-type C57BL/6 mice, nSREBP-1c transgenic mice, and *Tnf(-/-)* nSREBP-1c transgenic mice (31.3  $\pm$  2.6 g, 31.5  $\pm$  1.8 g, and

31.5  $\pm$  2.8 g, respectively). Glucose tolerance tests performed at the age of 20 weeks showed that nSREBP-1c transgenic mice had significantly higher plasma glucose levels than wild-type mice at each time point (► **Fig. 1a**). The glucose levels of *Tnf(-/-)* nSREBP-1c transgenic mice were significantly lower than those of nSREBP-1c transgenic mice, except at time 0. Fatty liver was observed in all nSREBP-1c Tg mice at the age of 20 weeks (► **Fig. 1c, e**). However, fatty deposition was milder and lower in prevalence in the livers from age-matched *Tnf(-/-)* nSREBP-1c Tg mice (20% vs. 100%, p<0.0001, ► **Fig. 1d, e**). Fibrosis was observed in the pericellular and perisinusoidal areas of the liver from nSREBP-1c Tg mice (► **Fig. 1c**). The frequency of liver fibrosis was significantly lower in *Tnf(-/-)* nSREBP-1c Tg mice than in nSREBP-1c Tg mice (15% vs. 65%, p=0.0057, ► **Fig. 1f**).



► **Fig. 3** The protein levels of TIMP1 **a** and NF-κB **b** in liver from wild-type mice, Tnf(-/-) mice, nSREBP-1c Tg mice, and Tnf(-/-) nSREBP-1c Tg mice at 20 weeks of age. Mean and SD (n=4, each). The immunohistochemical staining of TIMP1 in liver from a wild-type mouse **c**, a nSREBP-1c Tg mouse **d**, and a Tnf(-/-) nSREBP-1c Tg mouse **e** at 20 weeks of age. \* p<0.05, \*\* p<0.01,

### The expression of genes associated with lipid metabolism, inflammation, and fibrosis

nSREBP-1c Tg mice showed significantly higher expression of *Acaca*, *Scd1*, *Mcp1*, *Tgfb1*, *Col1a1*, and *Timp1* in the liver than wild-type mice (► **Fig. 2**). In contrast, Tnf(-/-) mice exhibited lower expression of *Scd1* and *Mcp1* in the liver. The hepatic expression of these 6 genes was significantly lower in Tnf(-/-) nSREBP-1c Tg mice compared with nSREBP-1c Tg mice.

The Western blot analysis showed that TIMP1 protein was increased in the liver from nSREBP-1c Tg mice (► **Fig. 3a, d**). However, the increase in TIMP1 protein was not observed in Tnf(-/-) nSREBP-1c Tg mice (► **Fig. 3a, e**). Similarly, the protein levels of NFκB were increased in the liver from nSREBP-1c Tg mice but not in the liver from Tnf(-/-) nSREBP-1c Tg mice (► **Fig. 3b**).

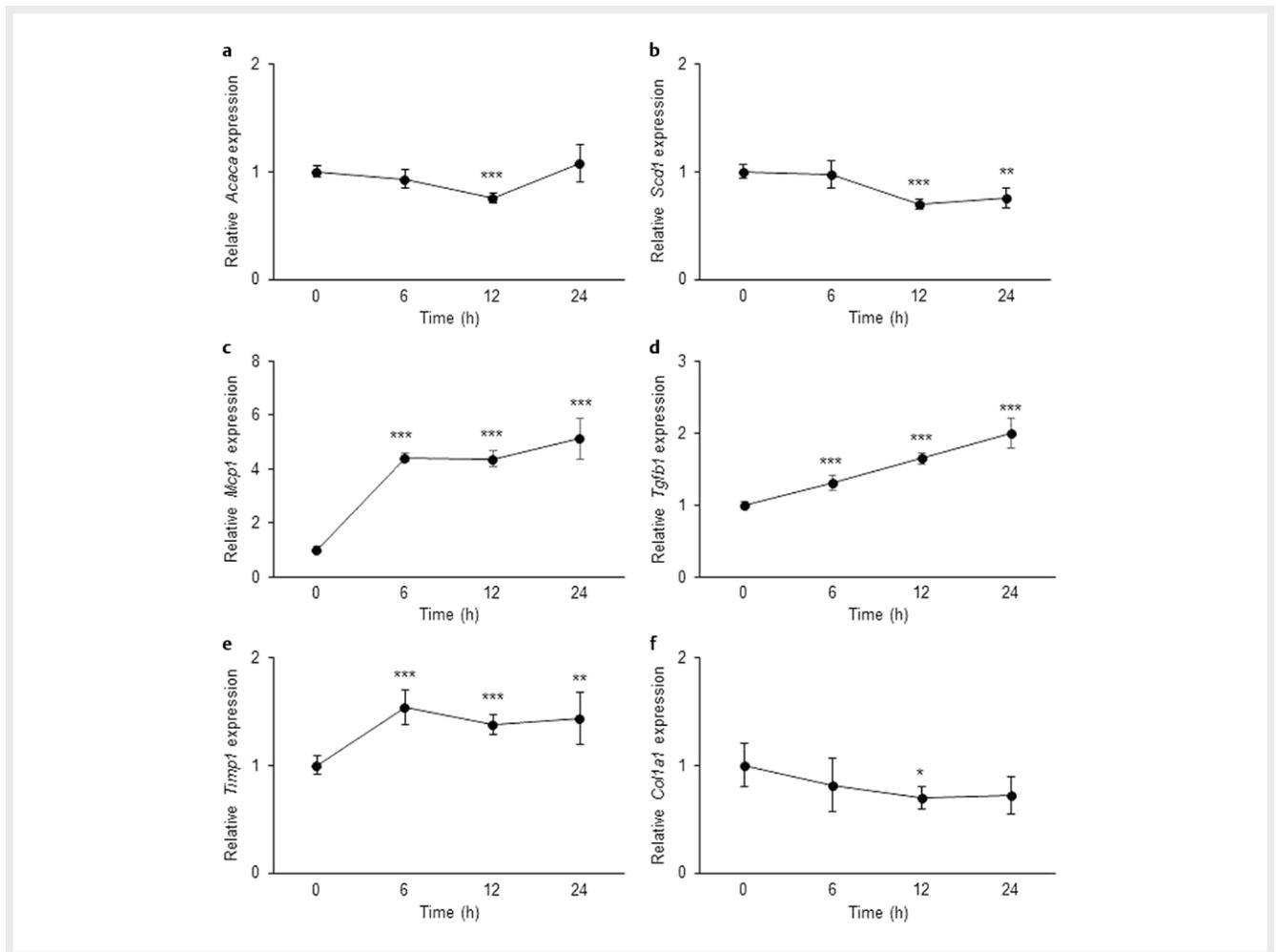
### Effects of TNF-α on primary cultured hepatocytes

Primary cultured hepatocytes of Tnf(-/-) mice were exposed to 20 ng/ml TNF-α for 6, 12, or 24 h. The expression of *Mcp1* increased more than four-fold at 6 h and remained elevated for up to 24 h (► **Fig. 4**). A similar pattern was observed in *Timp1* expression,

although the magnitude was low. The expression of *Tgfb1* was slightly but significantly elevated at 6 h, and it linearly increased for up to 24 h. In contrast, the expression of *Acaca*, *Scd1*, and *Col1a1* decreased rather than increased during the 24 h of incubation with TNF-α.

### Discussion

The excessive accumulation of hepatic triglycerides is caused by increased fatty acid delivery from peripheral adipose tissue to the liver and enhanced de novo lipid synthesis in the liver. Because the latter is likely the main mechanism of developing marked liver steatosis in lipodystrophic nSREBP1c Tg mice, we assessed the expression of genes for key enzymes in triglyceride synthesis. Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is the substrate for the biosynthesis of fatty acids [15]. In mammals, two ACC isoforms exist, ACC1 is cytosolic and participates in de novo lipogenesis in the liver and adipose tissue, while ACC2 is mitochondrial and is involved in the negative regulation of β-oxidation in the heart and skeletal muscle [16]. Stea-



**Fig. 4** The effects of TNF- $\alpha$  on the expression of Acaca **a**, Scd1 **b**, Mcp1 **c**, Tgfb1 **d**, Timp1 **e**, and Col1a1 **f** in primary cultured hepatocytes isolated from Tnf(-/-) mice. Hepatocytes were cultured in the presence of 20 ng/ml recombinant murine TNF- $\alpha$  for 6, 12, or 24 h. \*  $p < 0.01$  and \*\*  $p < 0.001$  vs. baseline. Mean and SD,  $n = 4$ .

royl-CoA desaturase is a rate-limiting enzyme in the synthesis of unsaturated fatty acids [17]. We analyzed the expression of the ACC1 gene, Acaca, and the Stearoyl-CoA desaturase gene, Scd1, and found that both genes were upregulated in nSREBP-1c Tg mice but not in Tnf(-/-) nSREBP-1c Tg mice, indicating that TNF- $\alpha$  is involved in the augmented de novo lipid synthesis in the liver of nSREBP-1c Tg mice. To determine whether TNF- $\alpha$  directly enhances lipid synthesis in the liver, we assessed the effect of TNF- $\alpha$  on the expression levels of Acaca and Scd1 in primary cultured hepatocytes. We used hepatocytes obtained from Tnf(-/-) mice to exclude the possible effect of endogenous TNF- $\alpha$ . However, exposure to TNF- $\alpha$  did not upregulate the genes in primary hepatocytes. As a result, TNF- $\alpha$  may be indirectly involved in the accumulation of triglycerides in the liver, which is probably through the induction of insulin resistance.

Chronic inflammation with mononuclear cell infiltration in the liver is a hallmark of NASH. Genes encoding chemokines and chemokine receptors are notably elevated in the livers of patients with NASH [18, 19]. In this study, we assessed the expression of Mcp1, which is the first discovered and most extensively studied

CC chemokine. In obesity, MCP-1 is secreted in high levels by hypertrophied adipocytes and plays a key role in promoting macrophage infiltration into adipose tissue [20, 21]. We found that Tnf(-/-) mice had lower levels of hepatic Mcp1 expression than wild-type mice, and the upregulation of Mcp1 expression in the liver of nSREBP1c Tg mice was attenuated by TNF- $\alpha$  deficiency. TNF- $\alpha$  may directly enhance Mcp1 production because Mcp1 expression was increased in primary hepatocytes exposed to TNF- $\alpha$ . It has been reported that TNF- $\alpha$  stimulates Mcp1 gene transcription through activating the Akt/PKB pathway [22]. As a result, TNF- $\alpha$ -induced Mcp1 expression may be an essential component in the progression of NAFLD to NASH and cirrhosis, although other inflammatory cytokines, including IL-1 $\beta$  [23] and IL-6 [24, 25], also induce MCP-1 production.

Next, we evaluated the expression of three genes associated with fibrosis. TGF- $\beta$  plays a central role in wound healing and tissue repair, and it is regarded as a key mediator in renal fibrosis. In chronic liver disease, TGF- $\beta$  activates stellate cells and fibroblasts, resulting in liver fibrosis [26]. Here we showed that the upregulation of TGF- $\beta$  expression in the liver of nSREBP-1c Tg mice was attenuated

by TNF- $\alpha$  deficiency. The *in vitro* studies using primary hepatocytes showed that TGF- $\beta$  expression was directly stimulated by exposure to TNF- $\alpha$ .

TIMP1, also called fibroblast collagenase inhibitor, is a natural inhibitor of matrix metalloproteinases, a group of peptidases involved in degrading the extracellular matrix [27]. It is a member of the family of four protease inhibitors, TIMP1, TIMP2, TIMP3, and TIMP4. Several studies have shown that TIMP1 is increased in liver fibrosis in both murine models and human samples, and it promotes liver fibrosis development [28–31]. It was shown in a bile duct ligation model that TNF- $\alpha$  may promote liver fibrosis via TIMP1 production from hepatic stellate cells [32]. Although TIMP1 is mainly thought to be secreted by stellate cells and Kupffer cells in the liver [33], it is also produced by hepatocytes under pathologic conditions, such as CCl<sub>4</sub>-induced liver fibrosis [34]. In this study, Timp1 expression was elevated in the liver of nSREBP-1c Tg mice compared with wild-type mice. However, Timp1 expression was markedly reduced in Tnf(-/-) nSREBP-1c Tg mice. Because TIMP1 protein levels were increased in the hepatocytes of nSREBP-1c Tg mice, we assessed the effect of TNF- $\alpha$  on the expression of Timp1 in primary cultured hepatocytes and demonstrated the upregulation of Timp1 expression in hepatocytes exposed to TNF- $\alpha$ . This indicates that TNF- $\alpha$ -induced Timp1 expression may be a key step in the development of NAFLD and progression to NASH. TGF- $\beta$  may be involved in the increase of Timp1 expression because Timp1 transcription is enhanced by the Smad signaling pathway downstream of the TGF- $\beta$  receptor [35].

Col1a1 encodes the pro- $\alpha$  1 chain of type 1 collagen, which is the most abundant collagen that forms collagen fibers, and it is the predominant collagen in cirrhotic livers [36, 37]. Col1a1 expression was markedly increased in the liver of nSREBP1c Tg mice, which is in accordance with the histological findings. The reduction of Col1a1 in Tnf deficient nSREBP1c Tg mice suggests that TNF- $\alpha$ -mediated chronic inflammation may be involved in the development of liver fibrosis. As for Timp1, Col1a1 is a downstream gene of the Smad signaling pathway [38–40]. However, Col1a1 expression was not increased in primary hepatocytes in response to TNF- $\alpha$  exposure. Other factors beyond TGF- $\beta$  may be required to augment Col1a1 gene expression.

In conclusion, TNF- $\alpha$  is involved in inducing key enzymes of lipid metabolism, inflammatory cytokines, and fibrosis-associated proteins in livers with steatosis. Hence, TNF- $\alpha$  likely plays a pivotal role in multiple steps of the development of NAFLD and progression to NASH. The attenuation of TNF- $\alpha$  action in the liver may help prevent or delay the development of NASH associated with metabolic syndrome.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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