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Expression and function of the atypical cadherin FAT1 in chronic liver disease

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ABSTRACT

Hepatic fibrosis can be considered as wound healing process in response to hepatocellular injury. Activation of hepatic stellate cells (HSCs) is a key event of hepatic fibrosis since activated HSCs are the cellular source of enhanced extracellular matrix deposition, and reversion of liver fibrosis is accompanied by clearance of activated HSCs by apoptosis. The atypical cadherin FAT1 has been shown to regulate diverse biological functions as cell proliferation and planar cell polarity, and also to affect wound healing. Here, we found increased FAT1 expression in different murine models of chronic liver injury and in cirrhotic livers of patients with different liver disease. Also in hepatic tissue of patients with non-alcoholic steatohepatitis FAT1 expression was significantly enhanced and correlated with collagen alpha I(1) expression. Immunohistochemistry revealed no significant differences in staining intensity between hepatocytes in normal and cirrhotic liver tissue but myofibroblast like cells in fibrotic septa of cirrhotic livers showed a prominent immunosignal. Furthermore, FAT1 mRNA and protein expression markedly increased during *in vitro* activation of primary human and murine HSCs. Together, these data indicated activated HSCs as cellular source of enhanced FAT1 expression in diseased livers. To gain insight into the functional role of FAT1 in activated HSCs we suppressed FAT1 in these cells by siRNA. We newly found that FAT1 suppression in activated HSCs caused a downregulation of NFκB activity. This transcription factor is critical for apoptosis resistance of HSCs, and consequently, we detected a higher apoptosis rate in FAT1 suppressed HSCs compared to control cells. Our findings suggest FAT1 as new therapeutic target for the prevention and treatment of hepatic fibrosis in chronic liver disease.

1. Introduction

Liver cirrhosis represents a major health problem with significant morbidity and mortality worldwide. Hepatic fibrosis develops in response to chronic liver disease of varying etiologies including viral hepatitis B and C infection, and alcoholic and nonalcoholic fatty liver disease. Progressive liver fibrosis ultimately leads to cirrhosis, which may cause organ failure and is a major risk factor for the development of hepatocellular cancer [1,2].

The hepatic scaring process is characterized by excessive accumulation of extracellular matrix (ECM) in response to chronic parenchymal damage. The key event of hepatic fibrosis is the activation of hepatic stellate cells (HSCs), which become the primary cellular source of ECM in diseased livers. In healthy livers, quiescent HSCs reside in the space of Disse, store retinoids, and produce factors that are trophic for parenchymal cells. Upon hepatic injury, HSCs transform to an activated myofibroblast-like phenotype, which is characterized by increased proliferation as well as proinflammatory and profibrogenic gene expression. Overgrowth of activated HSCs and their excessive ECM deposition are the driving force of progressive liver fibrosis. Conversely, regression of liver fibrosis occurs when activated HSCs are induced to undergo apoptosis [3,4].

Cell-cell adhesion is fundamental to multicellular architecture and integrity, and cadherins represent a major family of cell adhesion molecules [5]. In context of liver pathology, cadherins have mostly been studied in hepatocancerogenesis. E-cadherin attracted most attention, especially because of its role in EMT (epithelial-to-mesenchymal transition) and MET (mesenchymal-to-epithelial transition) [6]. An E-cadherin switch to N-cadherin has also been described during HSC activation [7], and it has been shown that loss of E-cadherin promotes up-regulation of transforming growth factor β1 and herewith facilitates liver fibrosis [8].

Abbreviations: HSCs, hepatic stellate cells; ECM, extracellular matrix; NASH, non-alcoholic steatohepatitis.

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The cadherin family has multiple subfamilies. Members of the FAT cadherin subfamily have an extraordinary large extracellular region, comprising 34 repeated domains, making them the largest cadherin molecules [9]. Classic FAT, identified in Drosophila, is known to regulate cell proliferation and planar cell polarity [10]. Furthermore, recent studies discovered FAT as a member of the Hippo signaling pathway controlling cell proliferation and survival [11]. FAT1 is one of four vertebrate orthologues and is engaged in several functions, including cell polarity and migration [12,13]. A homozygous knock out of FAT1 locus in mice was perinatal lethal by renal abnormalities [14].

The aim of the present study was to assess the expression and function of FAT1 in liver disease.

2. Materials and methods

2.1. Human tissue, cells and cell culture

Remnant liver samples were obtained from patients with informed consent through the Grosshadern Tissue Bank after partial hepatectomy. This tissue bank is regulated according to the guidelines of the non-profit state-controlled HTCR (Human Tissue and Cell Research) foundation following study approval [15]. Primary human hepatocytes (PHHs) and hepatic stellate cells (HSCs) were isolated and cultured as previously described [16]. *In vitro* activation of HSCs was achieved by cell culture on uncoated tissue culture dishes [17]. All experiments involving human tissues and cells have been carried out in accordance The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Animal models of hepatic injury

Bile duct ligation (BDL) was performed in mice for 3 weeks as described [18]. Sham surgeries were performed to obtain controls. Thioacetamide (TAA) induced liver fibrosis was induced by administering TAA (Sigma–Aldrich, Deisenhofen, Germany) at a dose of 300 mg/L in drinking water for 14 weeks [19]. Further, mice were fed with a non-alcoholic steatohepatitis (NASH) inducing diet [20] for 30 weeks. Liver tissue was immediately snap frozen and stored at −80 °C for subsequent analysis.

2.3. Transfection of hepatic stellate cells (HSCs)

Applying the HiPerfect transfection method (Qiagen, Hilden, Germany) HSCs were transiently transfected with small interfering RNA (siRNA; FAT1 siRNA-1 Hs_FAT_2, FAT1 siRNA-2 Hs_FAT_3, Qiagen). Transfection efficiency was determined by fluorescence-activated cell sorting (FACS) analysis applying Alexa Fluor 488-labeled control siRNA (AllStars negative control siRNA, Qiagen). Analysis revealed a transfection rate of approximately 80%.

For luciferase reporter assays, cells were transfected with 0.5 μg of a luciferase reporter construct harboring a NFκB-responsive element (Promega, Mannheim, Germany). To normalize transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega) was co-transfected and renilla luciferase activity measured by a luminometric assay (Promega) as described [21].

2.4. Expression analysis

Isolation of total cellular RNA from cultured cells and tissue and reverse transcription were performed as described [22]. Quantitative real time-PCR was performed applying LightCycler technology (Roche, Mannheim, Germany) [22] using the following sets of primers: FAT1 human (for: 5′-GTG TTT GTT CTC TGC CGT AAG-3′; rev: 5′-TAG GCT TCT GGA TGG AGT CG-3′), MCP1 human (for: 5′-CGC GAG CTA TAG AAG AAT CAC-3′, rev: 5′-TTG GGT TGT GGA GTG AGT G-3′), collagen alpha I(1) human (for: 5′-CGG CTC CTG CTC CTC TT-3′; rev: 5′-GGG GCA GTT CTT GGT CTC-3′). All other mRNA expression analyses were performed using QuantiTect Primer Assays according to the manufacturer's instructions (Qiagen).

2.5. Protein analysis

Protein extraction and Western blotting were performed as described [23] using following primary antibodies: anti-FAT1 (1:1000, Atlas Antibodies AB, Stockholm, Sweden), anti-α-sma (1:500, Abcam, Cambridge, UK), anti-collagen I (1:5000, Rockland, Gilbertsville, PA, USA), anti-IκBα (1:1000, Cell Signaling, Beverly, MA, USA), anti-β-actin (1:20,000, Sigma) and anti-α-tubulin (1:200, Santa Cruz, Santa Cruz, CA, USA). The membrane was stained with 0.5% Ponceau S to assure equal loading of the gel and transfer of the proteins [23,24].

For immunohistochemistry, standard 5 μm sections of formalin-fixed and paraffin-embedded tissue blocks were used. Immunohistochemical staining was performed using the polyclonal anti-FAT1 antibody (Atlas Antibodies AB) at a dilution of 1:50 as described [25].

2.6. Analysis of apoptosis

Apoptosis was induced in HSCs by staurosporine (STS) treatment (500 nM; 4 h) [26]. For detection of apoptosis, cells were stained simultaneously with FITC-conjugated Annexin V and propidium iodide (Promokine, Heidelberg, Germany) and analyzed by flow cytometry [17]. Furthermore, the Caspase-Glo 3/7 Assay (Promega) was used to analyze caspase-3/7 activity as described [27].

2.7. Statistical analysis

Results are expressed as mean ± standard error (range) or percent. Comparison between groups was made using the Student's unpaired *t*-test. Correlation between parameters was calculated with the Spearman test. A p value < 0.05 was considered statistically significant. All calculations were performed by using the GraphPad Prism Software (GraphPad Software, Inc., San Diego, USA).

3. Results

3.1. FAT1 expression in chronic liver disease

First, we analyzed FAT1 expression in three different murine models of chronic liver injury. After three weeks of bile-duct ligation (BDL) (Fig. 1A) as well as after 14 weeks of thioacetamide (TAA) application (Fig. 1B) hepatic FAT1 expression was significantly enhanced compared to control mice. Furthermore, hepatic FAT1 expression was significantly higher after feeding a non-alcoholic steatohepatitis (NASH) inducing diet compared to mice fed with standard chow (Fig. 1C). Also in cirrhotic human liver tissue of patients with alcoholic liver disease and chronic viral hepatitis B or C infection FAT1 expression was significantly higher compared to normal human liver tissue (Fig. 1D). In human NASH FAT1 expression revealed a high variation but significantly correlated ($r = 0.64$, $p = 0.002$) with the expression of collagen alpha I(1) (Fig. 1E), the most abundant extracellular matrix protein of fibrotic liver tissue. In contrast, no correlation was found between the expression of FAT1 and tumor necrosis factor (TNF) (Fig. 1F), a proinflammatory cytokine which is significantly elevated during liver inflammation. Immunohistochemistry revealed no significant

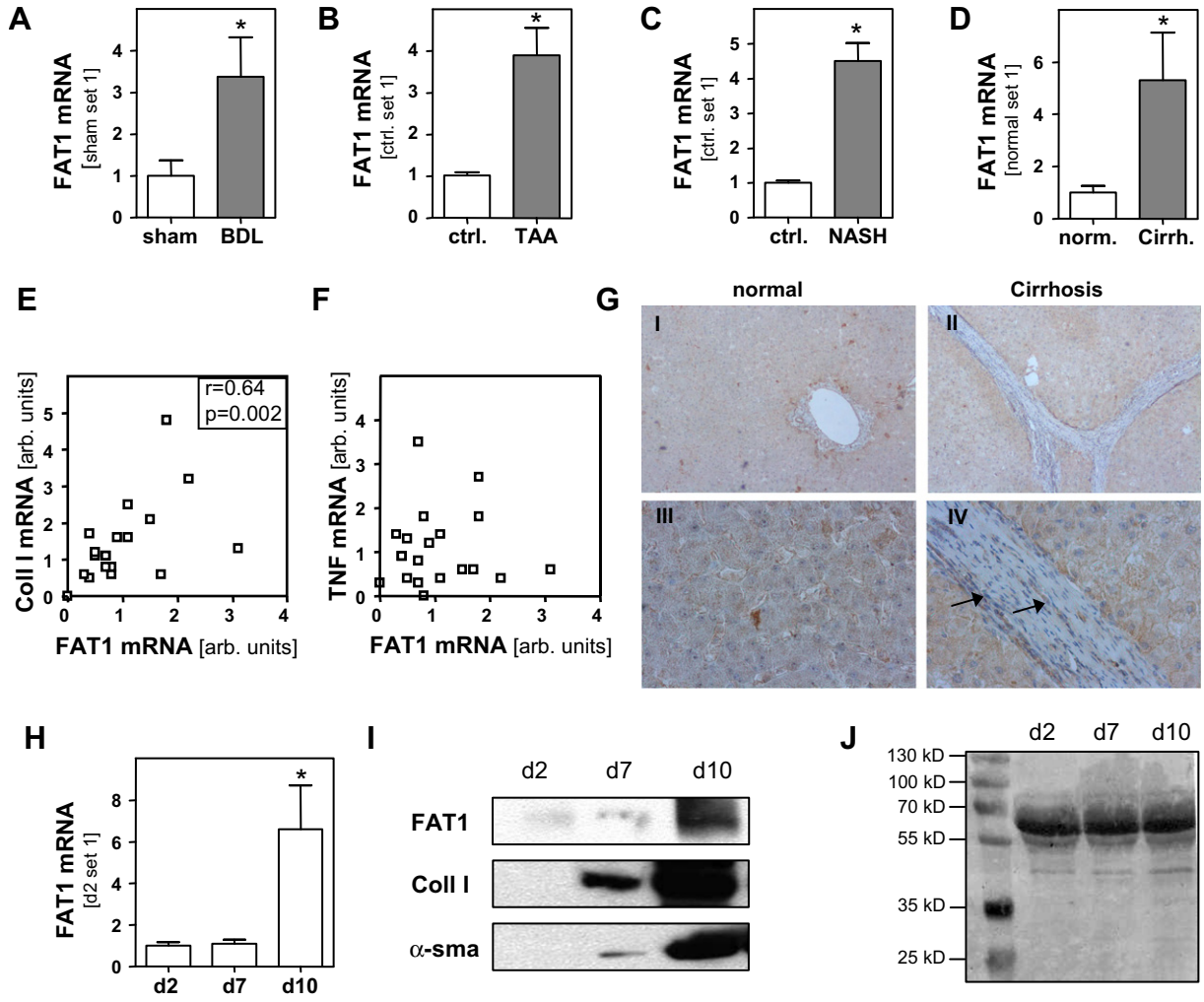

Fig. 1. FAT1 expression in chronic liver disease. Analysis of FAT1 mRNA expression in (A) bile-duct ligated (BDL) and sham-treated (sham) mice, (B) the murine TAA model, and (C) a dietary murine NASH model. (D) FAT1 mRNA expression in cirrhotic human liver tissue compared to normal liver tissue. Correlation of FAT1 with (E) collagen alpha I(1) (Coll I) and (F) tumor necrosis factor (TNF) mRNA expression in hepatic tissue of patients with non-alcoholic fatty liver disease. (G) Immunohistochemical analysis of FAT1 in normal (panel I, III) and cirrhotic liver tissue (panel II and IV). Myofiboblast like cells in fibrotic septa revealed a strong FAT1 immunosignal (IV; black arrows). Analysis of (H) FAT1 mRNA and (I) FAT1 protein expression during *in vitro* activation of human HSCs. Freshly isolated HSCs were cultured on plastic and RNA and cell lysates were isolated after different times of culture (day 2, 7 and 10). Collagen I (Coll I) and α-smooth muscle actin (α-sma) were used as activation markers. (J) Ponceau S staining of the same membrane was used for protein expression to demonstrate equal protein loading. (*$p < 0.05$).

differences in staining intensity between hepatocytes in normal and cirrhotic liver tissue (Fig. 1G). However, myofibroblast like cells in the fibrotic septa of cirrhotic livers revealed a prominent immunosignal. These data indicated activated HSCs as cellular source of enhanced FAT1 expression in diseased livers. In line with this, we observed a marked increase of FAT1 mRNA (Fig. 1H) and protein expression (Fig. 1I) during *in vitro* activation of primary human and murine (data not shown) HSCs. Collagen and alpha-smooth muscle actin are established markers of HSC activation [28] (Fig. 1I), and Ponceau S staining demonstrated equal protein loading (Fig. 1J).

3.2. Functional role of FAT1 in activated hepatic stellate cells

To gain insight into the functional role of FAT1 in activated HSCs, these cells were transiently transfected with specific FAT1 siRNA or control siRNA. Quantitative RT-PCR (Fig. 2A) and Western blot analysis (Fig. 2B) revealed a strong suppression of FAT1 in FAT1 siRNA transfected (FAT1 siRNA-1 and FAT1 siRNA-2) compared to control HSCs (ctrl. siRNA). Morphology did not differ between FAT1 siRNA and control siRNA transfected HSCs (Fig. 2C).

Expression analysis revealed significantly lower MCP-1 and Rantes mRNA levels in FAT1 suppressed HSCs (Fig. 3A). The expression of both chemokines is regulated by the transcription factor NFκB in activated HSCs [29]. In line with this, Western blot analysis revealed higher IκBα levels in FAT1 suppressed HSCs (Fig. 3B), indicative for reduced NFκB activity compared to control cells. Accordingly, an NFκB reporter gene assay confirmed reduced NFκB activity in FAT1 suppressed HSCs (Fig. 3C). In addition to its effect on proinflammatory gene expression, NFκB is critical for apoptosis resistance of activated HSCs [30]. Notably, caspase-3 activity was significantly higher in FAT1 suppressed HSCs compared to control cells (Fig. 3D) after incubation with 500 nM staurosporin (STS), which has been shown to induce apoptosis in activated HSCs [26]. In accordance with this, FACS analysis revealed a higher apoptosis rate in HSCs with suppressed FAT1 expression compared to control cells (Fig. 3E).

4. Discussion

Among vertebrate FAT cadherins, FAT1 is thus far the best studied and has been shown to be mainly expressed in proliferating

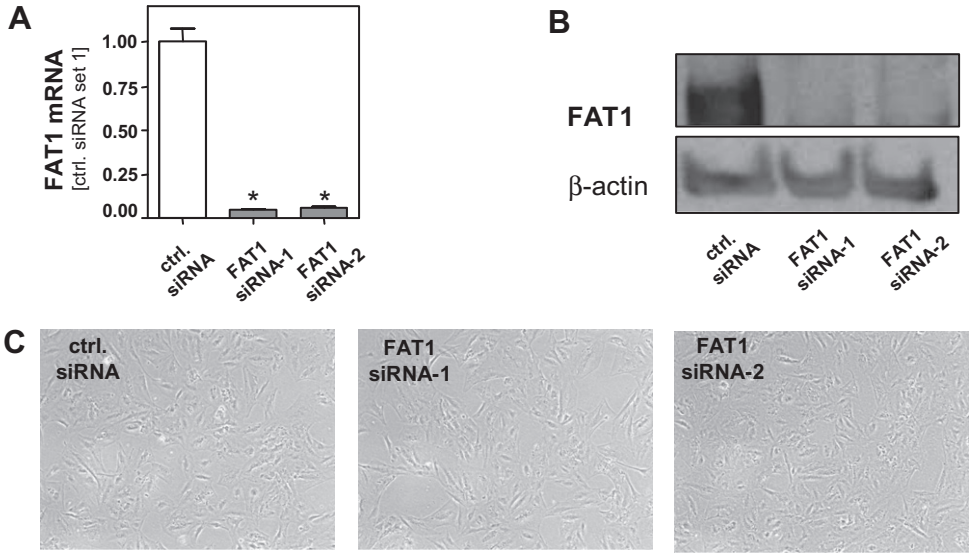

Fig. 2. FAT1 suppression in activated hepatic stellate cells (HSCs). Analysis of FAT1 in control siRNA (ctrl. siRNA) and FAT1 siRNA (FAT1 siRNA-1, FAT1 siRNA-2) transfected HSCs by (A) quantitative RT-PCR and (B) Western blotting. (C) Morphology of control and FAT1 suppressed HSCs. (*$p < 0.05$ compared to ctrl. siRNA).

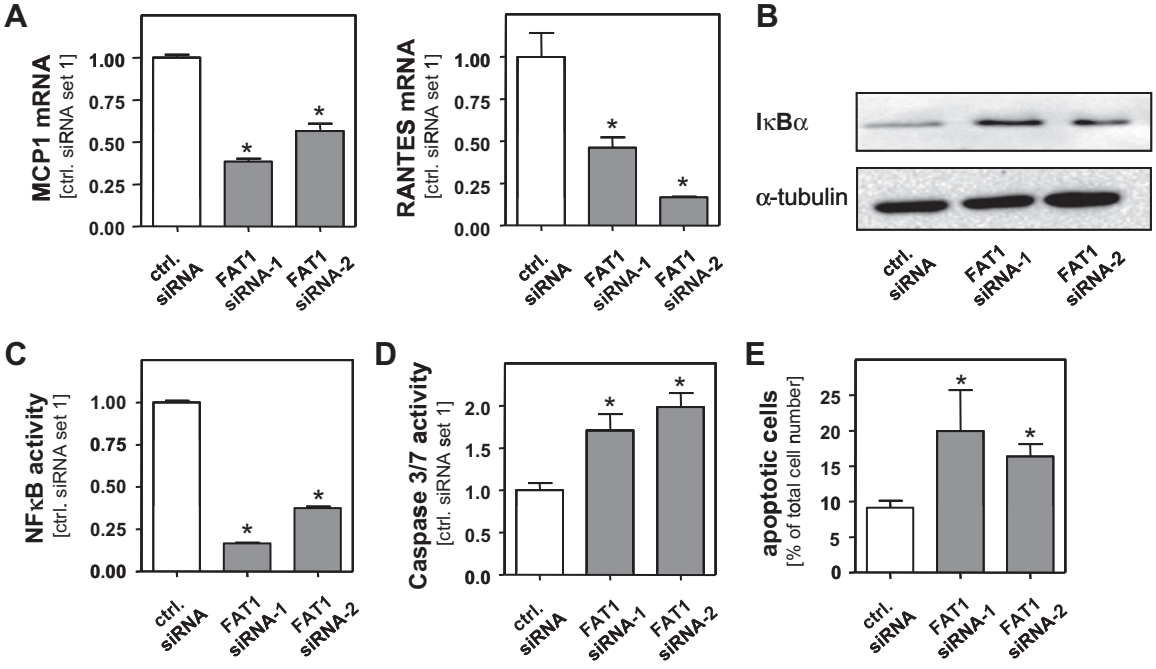

Fig. 3. Functional role of FAT1 in activated hepatic stellate cells (HSCs). (A) Effect of FAT1 suppression on MCP-1 and Rantes mRNA expression. (B) Western blot analysis of IκBα in FAT1 suppressed and control cells. (C) Reporter gene assay for NFκB activity in FAT1 suppressed and control HSCs. (D) Analysis of caspase-3/7 activity in FAT1 suppressed and control HSCs after STS (500 nM; 4 h) induced apoptosis. (E) Assessment of STS-induced apoptosis by flow cytometry applying annexin V and propidium iodide (PI) staining. Depicted is the mean-percentage of total apoptotic cells from 3 independent experiments (right panel). (*$p < 0.05$ compared to ctrl. siRNA).

epithelial tissue [9]. FAT1 expression peaks in the embryonic stage and diminishes later in the adult [9]. Mice lacking fat1 exhibit perinatal lethality by renal glomerulal abnormalities [14]. Further, FAT1 deficiency lead to defects in forebrain development and failure of eye development during embryogenesis [14] but no hepatic abnormalities have been described. Here, we aimed to assess the expression and function of FAT1 in liver disease. While in healthy adult liver hepatocytes reveal only a minimal replication rate, they start to proliferate in response to liver injury [31]. We observed a marked upregulation of FAT1 expression in different murine models of liver injury as well as in diseased human livers. Unexpectedly, immunohistochemistry revealed no significant increase of FAT1 expression in hepatocytes of diseased livers compared to healthy control livers. However and interestingly, a strong immunosignal was observed in myofibroblast-like cells in fibrotic septa of cirrhotic livers, and we found a strong upregulation in HSCs during *in vitro* activation. Moreover, hepatic FAT1 expression revealed a striking correlation with the expression of collagen alpha I(1), the major component of type I collagen. Upon hepatic injury this fibrillar collagen is produced by activated HSC and constitutes the most abundant extracellular matrix protein in fibrotic liver tissue. Together these data strongly indicate activated HSCs as the cellular source of increased FAT1 expression in diseased livers. A previous study showed increased FAT1 expression in injured arteries and

identified vascular smooth muscle cells as cellular source [32]. In vascular injury as well as in chronic kidney disease and an epithelial cell wound model FAT1 seems to be necessary for efficient wound healing [12,13]. Also, HSC activation and hepatic fibrosis can be considered as physiological wound healing process in response to acute hepatic injury. However, upon persistent hepatocellular injury excessive or dysregulated fibrosis leads to a distortion of the hepatic architecture and can lead to severe organ dysfunction. The correlation between FAT1 and collagen expression and the increased expression during HSC activation strongly suggest that this atypical cadherin also plays a critical role in hepatic fibrosis. FAT1 has been implicated in various biological processes [9] and in vascular smooth muscle cells it has been shown to affect cell polarization, migration and proliferation [32]. Here, we newly link FAT1 expression to apoptosis resistance in activated HSCs. We and others have shown that the activation of the transcription factor NFκB markedly increases during HSC activation and strongly contributes to the resistance to apoptosis in activated HSCs [23,30]. Notably, we found that suppression of FAT1 led to significant reduction of NFκB-activity in activated HSCs. This strongly indicates that the effect of FAT1 on apoptosis is at least in part mediated *via* NFκB activation.

Although fibrosis is a progressive pathological process there is emerging experimental and clinical evidence that even cirrhosis is potentially reversible. Key to this is the discovery that reversion of fibrosis is accompanied by clearance of activated HSC by apoptosis [3]. Consequently, there is considerable interest in determining the molecular events that regulate HSC apoptosis and the discovery of drugs that will stimulate HSC apoptosis in a selective manner. With regard to this, our study suggests FAT1 as an attractive therapeutic target for both prevention and treatment of hepatic fibrosis in chronic liver disease.

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