Overexpression of c-myc in hepatocytes promotes activation of hepatic stellate cells and facilitates the onset of liver fibrosis

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A R T I C L E   I N F O

Article history:
Received 4 March 2013
Received in revised form 28 May 2013
Accepted 3 June 2013
Available online 12 June 2013

Keywords:
Cell cycle
Hepatic stellate cell activation
Hepatocellular carcinoma
Liver fibrosis
Myofibroblast

A B S T R A C T

Background: Liver fibrosis is a consequence of chronic liver injury and can further progress to hepatocellular carcinoma (HCC). Fibrogenesis involves activation of hepatic stellate cells (HSC) and proliferation of hepatocytes upon liver injury. HCC is frequently associated with overexpression of the proto-oncogene c-myc. However, the impact of c-myc for initiating pathological precursor stages such as liver fibrosis is poorly characterized. In the present study we thus investigated the impact of c-myc for liver fibrogenesis.

Methods: Expression of c-myc was measured in biopsies of patients with liver fibrosis of different etiologies by quantitative real-time PCR (qPCR). Primary HSC were isolated from mice with transgenic overexpression of c-myc in hepatocytes (alb-myctg) and wildtype (WT) controls and investigated for markers of cell cycle progression and fibrosis by qPCR and immunofluorescence microscopy. Liver fibrosis in WT and alb-myctg mice was induced by repetitive CCl4 treatment.

Results: We detected strong up-regulation of hepatic c-myc in patients with advanced liver fibrosis. In return, overexpression of c-myc in alb-myctg mice resulted in increased liver collagen deposition and induction of α-SMA and further extracellular matrix proteins in vitro. Accordingly, fibrosis initiation in vivo after chronic CCL4 treatment was accelerated in alb-myctg mice compared to controls.

Conclusion: Overexpression of c-myc is a novel marker of liver fibrosis in man and mice. We conclude that chronic induction of c-myc especially in hepatocytes has the potential to prime resident HSC for activation, proliferation and myofibroblast differentiation.

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1. Introduction

The transcription factor c-myc orchestrates the expression of more than 15% of all human genes and thus mediates important biological effects including cell growth, proliferation, loss of differentiation and apoptosis [1–3]. One of the biological key functions of c-myc is its ability to promote cell cycle progression. In quiescent cells, c-myc expression is virtually undetectable. However, mitogenic stimulation of quiescent cells elicits a rapid rise in c-myc expression resulting in induction of G1-specific cyclin-dependent kinases including cyclin E-Cdk2 complexes and subsequent onset of DNA replication. Hence, ectopic overexpression of c-myc in experimental settings is frequently sufficient to drive quiescent cells into the progressive phase of the cell cycle [4–8]. C-myc is frequently deregulated or constitutively expressed in many human cancers [4]. In the liver, transgenic overexpression of c-myc in murine hepatocytes is sufficient to induce liver tumors although with high latency [9,10]. The gene signature of these tumors is similar to a group of slow-growing human hepatocellular carcinoma (HCC) [11].

HCC is one of the most common primary cancers and predominantly develops as a consequence of chronic liver injury in a multi-step process involving formation of liver fibrosis and cirrhosis. Hepatic fibrosis refers to the accumulation of extracellular matrix in response to acute or chronic liver injury from a wide variety of etiologies. A complex interplay among different hepatocyte types takes place during hepatic fibrogenesis. Hepatocytes are targets for most hepatotoxic agents, including hepatitis viruses, alcohol metabolites, and bile acids [12]. Damaged hepatocytes release pro-fibrotic signals and induce the recruitment of inflammatory cells, which eventually leads to activation of hepatic stellate cells (HSC). In response, resident quiescent HSC start to proliferate and transdifferentiate into a myofibroblast-like cell producing alpha-smooth-muscle-actin (α-SMA) and further extracellular matrix proteins [13–16]. Thus, re-generation of hepatocytes and activation of HSCs during chronic liver injury involves transition of these normally quiescent cells into the
cell cycle leading to cell proliferation. We recently demonstrated that the cell cycle mediator cyclin E1 is essential for proliferation and activation of HSC but less relevant for proliferation of regenerating hepatocytes [17,18]. However, the role of c-myc – which is thought to act upstream of cyclin E1 – for initiation and progression of liver fibrosis is largely unknown.

Here, we investigated the contribution of c-myc for hepatic fibrogenesis. We show that c-myc is over-expressed in livers of patients and mice with advanced liver fibrosis and provide further evidence that chronic c-myc expression especially in hepatocytes predisposes to accelerated fibrogenesis through crosstalk with HSC.

2. Materials and methods

2.1. Human liver samples

Human liver samples were available from routine liver biopsies or from explanted cirrhotic livers resulting from transplantation, as described recently [19] and are further characterized in Supplementary Table 1. Grading and staging of all liver specimens were performed according to Desmet–Scheuer score by a pathologist who was blinded for experimental data. Liver samples with signs of HCC were excluded from further analysis. Control liver samples without fibrosis were obtained from unaffected areas of liver resections for secondary liver malignancy (mostly metastasis of colorectal cancer). The study protocol was approved by the ethics committee of University Hospital Aachen, and conducted according to the principles expressed in the Declaration of Helsinki. Human liver tissues were either subjected to RNA isolation or paraffin embedded and stained with a c-myc antibody (Abcam, Cambridge, UK).

2.2. Maintenance and treatment of mice

All animals were maintained in the animal facility of the University Hospital Aachen in a temperature-controlled room with 12-hour light/dark cycle. Animal husbandry and procedures were approved by the authority for environment conservation and consumer protection of the state North Rhine-Westfalia (LANUV, Germany). For our study we used male transgenic mice carrying a c-myc transgene under the control of glutamine and 4 mmol/l penicillin/streptomycin (PAA, Pasching, Austria). Well and cultivated in DMEM supplemented with 10% FCS, 4 mmol/l L-glutamine and 4 mmol/l penicillin/streptomycin. Conditioned medium (CM) was collected from hepatocytes grown in DMEM supplemented with 0.2% FCS for 24 h in order to minimize interference with factors present in serum.

2.3. Isolation of hepatic stellate cells (HSC) and primary hepatocytes

HSC were isolated from adult male mice in a C57BL/6 background weighting approximately 25 g following the collagenase method as described recently [17]. Freshly isolated HSC were washed and plated in 6-well plates (Falcon) at a density of 150,000 cells per well and cultivated in DMEM supplemented with 10% FCS 4 mmol/l L-glutamine and 4 mmol/l penicillin/streptomycin (PAA, Pasching, Austria). Primary mouse hepatocytes were isolated from 7 to 8-week-old mice by collagenase perfusion [21]. Live cells were plated on collagen type 1 coated petri dishes at a density of 1.2 x 10^5/cm² in DMEM medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with L-glutamine, high glucose (4.5 g/l) and 100 U/ml penicillin/streptomycin. Conditioned medium (CM) was collected from hepatocytes grown in DMEM supplemented with 0.2% FCS for 24 h in order to minimize interference with factors present in serum.

2.4. Histological evaluation and immunofluorescence stainings of liver sections and isolated HSC

Immunofluorescence staining of HSC on cover slips was performed according to our recent report [17] using primary antibodies for α-SMA (Sigma, Steinheim, Germany), Ki-67 (Dako, Glostrup, Denmark), c-myc and cyclin E (Santa Cruz, CA). All fluorescence-labeled cells were analyzed and documented using an Imager Z1 fluorescence microscope together with Axiosview software (Carl Zeiss, Jena, Germany). Hepatic tissues were fixed in 4% paraformaldehyde (PFA) immediately after extraction, embedded in paraffin, sectioned and subjected to staining for H&E, Sirius red and Ki-67 as described recently [17]. Immunostaining of Platelet-derived Growth Factor B (PDGF-B) was performed using an Anti-PDGF BB antibody from Abcam (Cambridge, UK).

Quantitative determination of liver fibrosis in mice was assessed histologically by quantification of Sirius red-positive areas as described recently [22]. Briefly, a minimum of 10 photographs of random high-power fields (100 x magnifications) were taken of each Sirius red-stained liver section. Pictures containing large bile ducts or vessels were excluded from further analysis. Images were converted to greyscale and subjected to signal quantification using free NIH ImageJ software (http://imagej.nih.gov/ij/, National Institutes of Health, Bethesda, MD) according to the provider’s instructions.

2.5. RNA isolation and quantitative real-time PCR analysis (qPCR)

The procedure to isolate total RNA and perform qPCR was performed as described recently [17]. All measurements were normalized using GAPDH expression as an internal standard and calculated as fold induction in comparison to untreated controls. The primer sequences are indicated in Supplementary Table 2.

2.6. Immunoblot analysis

Western blots were carried out according to standard procedures [17]. Membranes (Whatman® Protran®) were probed with antibodies for c-myc (Santa Cruz), α-SMA (Sigma, Steinheim, Germany), collagen 1 (PS065 Monosan, Beutelsbach, Germany) and PCNA (Invitrogen). As secondary antibodies, anti-rabbit-HRP (Cell Signaling) and anti-mouse-HRP (Santa Cruz) were used. GAPDH probing was performed as internal control using an antibody from AbD serotec (Kidlington, UK).

2.7. Statistical analysis

Data are expressed as mean ± standard deviation of the mean. Statistical significance was determined by two-way analysis of variance followed by a Student’s t test.

3. Results

3.1. Liver fibrosis in patients is associated with induction of c-myc

We determined c-myc expression in liver biopsies from patients with liver fibrosis of different etiologies (see Supplementary Table 1). Hepatic c-myc mRNA expression was approximately tenfold up-regulated in patients with advanced (F3) liver fibrosis and also significantly increased in patients with liver cirrhosis (F4, Fig. 1A). In line with these findings, immunohistological analysis of these samples (F3, F4) revealed a significant increase in c-myc expression especially in hepatocytes but also in non-parenchymal liver cells (Fig. 1B). Thus, c-myc is deregulated in the liver during fibrogenesis, which might be important for disease progression.
3.2. Overexpression of c-myc in hepatocytes promotes liver fibrosis

The role of c-myc for the initiation and progression of liver fibrosis was further investigated using transgenic mice over-expressing c-myc in hepatocytes (alb-myc\textsuperscript{Gt}). These mice resemble a well-established animal model of hepatocarcinogenesis characterized by low proliferation, high apoptosis and development of slow-growing liver tumors with long latency [23]. Accordingly, in 45-week-old alb-myc\textsuperscript{Gt} mice less than 40% of the animals developed spontaneous HCCs [9]. Hepatic c-myc mRNA expression in these mice showed high variation ranging from 40 to 200-fold expression over baseline. However c-myc protein was detected in all investigated mice between 10 and 65 weeks of age (Supplementary Fig. 1A–B).

We next tested spontaneous proliferation and apoptosis in the liver of alb-myc\textsuperscript{Gt} mice at different age. Of note, mature alb-myc\textsuperscript{Gt} mice revealed constitutive expression of the positive cell cycle mediator Proliferating-Cell-Nuclear-Antigen (PCNA) and simultaneous activation of the pro-apoptotic protein caspase-3 (Fig. 2A). Increased cell cycle activity in hepatocytes and non-parenchymal cells of alb-myc\textsuperscript{Gt} mice was confirmed by demonstrating elevated numbers of Ki-67 positive cells (indicative of overall cell cycle activity), and by the up-regulation of positive cell cycle mediators such as pRb, cyclin E1 and cyclin A2 (Supplementary Fig. 1C–E). Despite detectable caspase-3 activation in alb-myc\textsuperscript{Gt} mice, we observed a rather modest liver apoptosis in these mice at all age cohorts investigated (Supplementary Fig. 2A). However, liver transaminases in alb-myc\textsuperscript{Gt} mice were only slightly but not significantly elevated in younger mice (until the age of 35 weeks), while older mice showed a stronger induction of transaminase values presumably due to the first appearance of liver tumors (Supplementary Fig. 2B). We therefore concluded that mild chronic liver injury (i.e. apoptosis) in alb-c-myc\textsuperscript{Gt} mice may trigger a constant compensatory proliferation of surrounding hepatocytes.

Remarkably, liver tissue of alb-myc\textsuperscript{Gt} mice exhibited spontaneous fibrillar collagen deposition from 10 weeks onward, as detected by Collagen I staining (Fig. 2B–C). The extent of hepatic collagen expression was further increased in an age-dependent manner and was substantially higher in transgenic mice compared to WT animals of the same age. Of note, the collagen staining pattern did not reflect true liver fibrosis. However, gene and protein expression of the fibrosis marker alpha-smooth-muscle-actin (\(\alpha\)-SMA, predominantly expressed in activated HSC) was significantly higher in alb-myc\textsuperscript{Gt} mice of all age cohorts in comparison to WT controls (Fig. 2D–E). Taking together increased hepatic collagen accumulation and HSC activation in alb-myc\textsuperscript{Gt} mice indicates a predisposition to liver fibrosis due to c-myc overexpression.

3.3. Overexpression of c-myc in hepatocytes is associated with alterations in hepatic metabolism and induction of PDGF-B expression

It has been previously shown that c-myc can regulate cellular metabolism by transcriptional up-regulation of target genes involved in glucose uptake and lactate production such as Lactate Dehydrogenase A (LDH-A) [24,25]. Since a recent study suggested that accumulation of lactate in liver has pro-fibrotic properties [26], we evaluated whether the increased collagen deposition in alb-myc\textsuperscript{Gt} mice was related to c-myc-dependent deregulation of the hepatic metabolism. To this end, we measured the gene expression of key enzymes involved in glycolysis and lipogenesis as illustrated in Fig. 3A. Unexpectedly, in alb-myc\textsuperscript{Gt} mice we did not detect aberrant expression of Pyruvate Kinase M1 (PKM1), which usually inactivates the enzyme responsible for conversion of pyruvate to acetyl-CoA. In addition, we observed down-regulation of LDH-A in c-myc transgenic mice suggesting that in our experimental setting c-myc expression does not result in elevated lactate production (Fig. 3B). However, c-myc overexpression inhibited lipogenesis-related genes as demonstrated by the down-regulation of the Fatty Acid Binding Transporter 4 (FATP4) and the Peroxisome Proliferator-activated Receptor gamma (PPARY). Of note, depletion of PPAR\(\gamma\) is related to enhanced liver fibrogenesis as reported earlier [27].

We next investigated potential paracrine effects of c-myc overexpressing hepatocytes in situ as this could explain improved activation of fibrogenic cells in alb-myc\textsuperscript{Gt} mice. Platelet-derived growth factor B (PDGF-B) is one of the key players in development of hepatic fibrosis [15]. Interestingly, we detected cytoplasmic expression of PDGF-B in hepatocytes from alb-myc\textsuperscript{Gt} mice which was strong in old animals and less frequent in young mice (Fig. 3C). In summary, overexpression of c-myc in hepatocytes induces pro-fibrotic signals such as down-regulation of PPAR\(\gamma\) and expression of PDGF-B.

3.4. Enhanced cell cycle progression and myofibroblast-transdifferentiation of HSC from alb-myc\textsuperscript{Gt} mice

We were next interested to define the cellular mechanism leading to collagen deposition in alb-myc\textsuperscript{Gt} livers and focussed on HSC as a
potential effector cell population. We isolated HSC from tumor-free alb-myc\textsuperscript{ck} mice and from WT controls of the same age (45 weeks old) and measured c-myc and α-SMA mRNA expression. Of note, HSC derived from WT or alb-myc\textsuperscript{ck} mice showed the same basal c-myc expression level (Supplementary Fig. 3A), which was expected since the c-myc transgene was expressed specifically in hepatocytes. However, basal α-SMA levels were significantly up-regulated in freshly isolated HSCs from alb-myc\textsuperscript{ck} animals (Fig. 4A) suggesting that the liver of alb-myc\textsuperscript{ck} mice contains a population of pre-activated HSC.

To study this phenomenon in more details we investigated cell cycle progression, transdifferentiation and survival of HSC from WT and alb-myc\textsuperscript{ck} mice in vitro over a time period of ten days (d1–d10). At the beginning of the experiments both HSC populations were comparable regarding density and purity as confirmed by microscopy and visualization of intracellular vitamin A droplets following UV excitation (Supplementary Fig. 3B).

HSC from alb-myc\textsuperscript{ck} mice revealed accelerated transactivation compared to controls beginning already at day 3 after seeding. After 7 days, the majority of HSC from transgenic animals had a fibroblast-like morphology and reached confluence (Fig. 4B). Our findings were confirmed by gene expression analysis for α-SMA and collagen I. In HSC from transgenic mice, overall α-SMA and collagen expression significantly exceeded WT levels at all investigated time points (Fig. 4C–D). To monitor cell cycle progression and transdifferentiation of HSC into myofibroblasts, we performed co-staining for Ki-67 and α-SMA. In WT HSC, around 5% of total HSC were Ki-67 positive at day 4 after seeding (Fig. 5A). In contrast, Ki-67 expression was detectable in more than 15% of HSC from alb-myc\textsuperscript{ck} mice suggesting enhanced cell cycle activity of these cells (Fig. 5A–B).

C-myc overexpression in alb-myc\textsuperscript{ck} mice was restricted to hepatocytes. Accordingly, HSC derived from alb-myc\textsuperscript{ck} mice showed an identical c-myc expression profile compared to WT HSC revealing transient c-myc induction limited to day 1 after plating (Fig. 5C). In our previous work [17] we demonstrated that cell cycle progression, myofibroblast-transdifferentiation and survival of HSC depends on cyclin E1. In turn, c-myc controls the activation of the cyclin E/Cdk2 kinase complex resulting in cell proliferation. In fact, transient c-myc induction in WT HSC preceded the induction of cyclin E1 in these cells (Fig. 5D). We thus compared cyclin E1 mRNA expression levels in HSC from WT and alb-myc\textsuperscript{ck} mice throughout the transdifferentiation process. In line with our previous finding, cyclin E1 expression was significantly elevated in HSC from transgenic mice at days 3, 4 and 7 after plating (Fig. 5E).

Using immunofluorescence microscopy, we could also demonstrate the enhanced expression of cyclin E1 in nuclei of HSC from alb-myc\textsuperscript{ck} mice already at day 3 after plating (Fig. 5F). From this data we conclude that overexpression of c-myc in hepatocytes has the potential to prime resident HSC for activation and proliferation while c-myc induction in HSC is sharply restricted to an early phase of activation and does not influence cyclin E1 expression.

One possible mechanism for HSC pre-activation through c-myc expressing hepatocytes could be the c-myc-driven secretion of profibrogenic mediators such as PDGF-B as already demonstrated in situ (compare Fig. 3C). To further support this hypothesis, we stimulated primary WT HSC with conditioned medium derived either from cultured alb-myc\textsuperscript{ck} hepatocytes (CM alb-myc\textsuperscript{ck}) or WT hepatocytes (CM WT) and analyzed the kinetics of HSC transactivation in vitro. However, in this experiment we did not detect morphological differences between CM alb-myc\textsuperscript{ck} and CM WT treated HSC within the observation time of 10 days (Supplementary Fig. 4A). In line with this finding, induction of α-SMA occurred with the same kinetics in both groups (Supplementary Fig. 4B–C). Interestingly, treatment of WT HSC with conditioned hepatocyte-derived medium per se accelerated the induction of α-SMA and collagen I (Supplementary Fig. 4D–H). However, these effects were c-myc independent and could also be achieved with CM WT to the same extend. In summary, these experiments suggest that the crosstalk

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**Fig. 2.** Age-dependent hepatic collagen deposition in alb-myc\textsuperscript{ck} mice. Tumor-free alb-myc\textsuperscript{ck} mice and matching WT littermate controls at the age of 10 (n = 7 per group) or 65 weeks (n = 7 per group) were analyzed for markers of proliferation, apoptosis and liver fibrosis. (A) Protein expression of PCNA and cleaved caspase-3 demonstrating elevated proliferation and apoptosis in aged alb-myc\textsuperscript{ck} liver. (B) Detection of basal collagen deposition (collagen type I α1, stained in red) in liver of WT and alb-myc\textsuperscript{ck} mice at the age of 10 weeks (10w, upper panel) and 65 weeks (65w, lower panel) using immunofluorescence microscopy. (C) Quantification of collagen I stained tissue area using image analysis software. (D) Quantification of hepatic α-SMA mRNA expression. (E) Determination of α-SMA protein expression in 65-week-old WT and alb-myc\textsuperscript{ck} mice. *: p < 0.05; **: p < 0.01; ***: p < 0.001.
Fig. 3. Overexpression of c-myc in hepatocytes is associated with alterations in hepatic metabolism and induces expression of PDGF-B. (A) Schematic overview of the analyzed metabolic pathways. Left: Glycolysis under hypoxic conditions resulting in lactate production. PKM1: Pyruvate kinase M1; LDH-A: Lactate Dehydrogenase A. Right: Glucose metabolism and lipogenesis under normoxic conditions. TCA cycle: Tricarboxylic Acid cycle; FATP: Fatty Acid binding transporter; PPARγ: Peroxisome proliferator-activated receptor gamma. (B) Liver samples from WT and tumor-free alb-myctg mice at the age of 65 weeks (n = 7 per group) were analyzed for expression of genes involved in glycolysis (LDH-A, PKM1) and lipogenesis (PPARγ, FATP4) by qPCR. *: p < 0.05; ***: p < 0.001; n.s.: not significant. (C) Paraffin liver sections from WT and tumor-free alb-myctg mice at the age of 10 and 55 weeks were stained with an antibody for PDGF-B. Hepatocytes with cytoplasmic expression of PDGF-B are stained in brown (arrows).
of c-myc expressing hepatocytes and HSC depends on an intact tissue environment and cannot be mimicked in vitro.

3.5. Overexpression of c-myc in hepatocytes accelerates liver fibrogenesis during chronic CCl₄ treatment

We next investigated the involvement of c-myc in experimental murine liver fibrosis. To this end, we subjected WT mice to periodical carbon tetrachloride (CCl₄) injections for up to 4 weeks. In agreement with our findings in human samples, c-myc mRNA and protein expression in murine liver was strongly induced after CCl₄ treatment and correlated with fibrosis progression as determined by α-SMA quantification (Fig. 6A–B). Immunohistochemistry revealed that following CCl₄-treatment the c-myc protein accumulated in both non-parenchymal cells and hepatocytes while c-myc could not be detected in untreated control samples (Fig. 6C).

Based on our previous results, we hypothesized that c-myc overexpression of hepatocytes in vivo could lead to an enhanced propensity of mice to develop liver fibrosis by facilitating HSC activation. Therefore, we investigated the consequences of chronic
CCl₄-treatment in alb-myctg mice in comparison to WT controls. Repeated injections of CCl₄ for 2 weeks induced only minor collagen expression in WT livers, whereas in livers from alb-myctg mice bridging fibers were already detectable in Sirius red stained tissue sections (Fig. 7A–B). These findings were confirmed by qPCR showing approximately 3 times increased expression of hepatic α-SMA in alb-myctg mice in comparison to WT controls (Fig. 7C). We also performed expression analysis of pro-fibrotic proteins confirming accelerated onset of α-SMA and collagen I expression in alb-myctg (Fig. 7D). In addition, PCNA was also prematurely induced indicating enhanced proliferation of hepatic cells in alb-myctg mice (Fig. 7D). Consistently, immunohistological analysis of liver sections confirmed increased proliferation of hepatic cells in alb-myctg animals as evidenced by Ki-67 staining (Fig. 7E).

A more detailed analysis demonstrated that in alb-myctg mice hepatocytes are more prone to proliferation compared to WT cells, while proliferation of non-parenchymal cells was similar in WT- and alb-myctg liver (Fig. 7F). In agreement with our previous observations, the gene and protein expression of c-myc after 2 weeks of CCl₄ administration was significantly stronger in the liver from transgenic mice compared to WT animals (Fig. 7G–H). Our findings are summarized in Fig. 8 and implicate that pre-activation of HSC by c-myc overexpressing hepatocytes primes the liver for accelerated fibrosis induction at least in the CCl₄ model.
4. Discussion

Initiation and progression of liver fibrosis is related to proliferation of hepatic cells such as hepatocytes and HSC. As a consequence of chronic liver injury and hepatocyte death, remnant hepatocytes leave their quiescent state and re-enter the cell cycle in order to restore the original liver mass and function. Hepatocyte death also stimulates the proliferation and activation of HSC resulting in transdifferentiation and matrix production. We recently demonstrated that the cell cycle mediator cyclin E1 is essential for activation, proliferation and survival of HSC during liver fibrogenesis [17] thereby highlighting the yet underestimated role of the cell cycle machinery for initiation of liver fibrosis. Cyclin E1 is under control of the proto-oncogene c-myc [28]. Although it is well established that gene amplification and overexpression of c-myc is associated with development of hepatocellular carcinoma [29–31], the time point of c-myc induction, but also its role for HSC precursor stages such as fibrosis is poorly characterized.

Here, we demonstrated for the first time that c-myc is over-expressed in patients with advanced liver fibrosis but also in experimental liver fibrosis in mice. Additionally, transgenic overexpression of c-myc exclusively in hepatocytes triggered increased hepatic collagen deposition and was sufficient to prime wildtype-like HSC with normal c-myc expression for accelerated activation. As a consequence, transgenic mice with overexpression of c-myc in hepatocytes showed strongly accelerated onset of liver fibrosis following chronic injury.

Our results raised the question whether liver fibrosis is rather triggered by endogenous c-myc induction as a direct response to liver injury or by constitutive c-myc expression due to acquired genetic instability and gene amplification. Of note, c-myc amplification was described in 30%–80% of primary HCC in several studies [31–33]. We mimicked c-myc amplification by using transgenic alb-myc<sup>ES</sup> mice, which is a well-established animal model of hepatocarcinogenesis [30]. However, continuous overexpression of c-myc exclusively in hepatocytes resulted in only moderate HCC incidence of approximately 30%–60% within one year [9,30]. From our own data in alb-myc<sup>ES</sup> mice we conclude that gene amplification of c-myc in hepatocytes alone is not sufficient to induce true liver fibrosis, but rather provides a pro-fibrotic tissue environment through basal moderate apoptosis, pre-activation of HSC and high basal collagen expression eventually leading to stronger fibrogenesis after stimulation via a second hit. In contrast, our data from CCL<sub>2</sub>-treated WT mice clearly indicated that chronic liver injury is associated with induction of endogenous c-myc. Interestingly, c-myc expression levels did not correlate with fibrosis stage but were maximal at a medium phase (F3 in patients; 2 weeks after CCl<sub>4</sub> treatment in mice) of fibrosis progression further suggesting that regulated c-myc induction per se, but not c-myc gene amplification and constitutive expression, is a prerequisite for fibrosis induction.

Another relevant matter in our study was the identification of the major target cell for c-myc mediated pro-fibrotic processes. Our in situ analysis in human fibrotic livers revealed c-myc expression in both hepatocytes and non-parenchymal cells such as HSC. However, during HSC activation we detected only early and transient c-myc induction which was identical in WT cells and pre-activated HSC derived from alb-myc<sup>ES</sup> mice. Moreover, we still detected strong cyclin E1 expression, HSC proliferation and collagen production after c-myc was turned off and remarkably, cyclin E1 expression was even higher in HSC from alb-myc<sup>ES</sup> mice in the absence of detectable c-myc gene expression. We therefore conclude that c-myc is completely dispensable for cyclin E1 expression, cell cycle progression and transactivation of HSC but is relevant as a trigger for early HSC activation.

Accordingly, c-myc expression in hepatocytes seems to be a major trigger of liver fibrosis. Our data clearly demonstrated that continuous c-myc expression such as in alb-myc<sup>ES</sup> mice resulted in moderate but chronic hepatocyte apoptosis and pre-activated endogenous HSC for accelerated proliferation and transdifferentiation induced by a second stimulus. We therefore hypothesize that early acquisition of c-myc amplification would predispose patients with chronic hepatitis for accelerated onset of severe liver fibrosis and cirrhosis.

Mechanistically, our data point a crosstalk between c-myc expressing hepatocytes and HSC through several independent signals. Recent studies suggested that changes in the hepatic metabolism such as inhibition of lipogenesis and lactate accumulation due to aerobic glycolysis may contribute to HSC activation [26]. We initially hypothesized that alb-myc<sup>ES</sup> mice could be predisposed to liver fibrosis due to enhanced pro-fibrotic...
lactate production as described for a similar transgenic mouse model with myc-expression under control of the Phosphoenolpyruvate carboxykinase (PEPCK) promoter [25]. However our data clearly excluded this possibility. Instead, c-myc overexpression largely prevented hepatic PPARγ expression. Of note, PPARγ-deficient mice are prone to enhanced liver fibrosis after injury [27]. We thus conclude that c-myc mediated inhibition of lipogenesis and down-regulation of PPARγ may contribute to pre-activation of HSC in Alb-mycTG mice.

As a second potential mediator for the crosstalk between c-myc expressing hepatocytes and HSC we identified PDGF-B, which is one of the key fibrogenic stimuli to HSC activation. We detected obvious cytoplasmic PDGF-B expression exclusively in hepatocytes of Alb-mycTG mice which increased with age. This was unexpected since hepatocytes are usually not the main source of PDGF in the liver. However, previous studies have demonstrated that transgenic expression of PDGF-B selectively in hepatocytes increases hepatic collagen deposition in mice and predisposes to enhanced liver fibrosis after CCl4 treatment [34] reflecting several properties of Alb-mycTG mice. Therefore our data strongly suggest that PDGF-B expression is induced in hepatocytes by c-myc and allows for paracrine signaling on neighboring HSC contributing to their activation. However, we could not mimic these postulated effects in vitro. Although our experiments using supernatants of hepatocyte cultures in vitro showed that paracrine mechanisms in hepatocytes are able to drive HSC activation, we were not able to detect c-myc

Fig. 7. Overexpression of c-myc in hepatocytes triggers accelerated liver fibrogenesis during chronic CCl4 treatment. Tumor-free Alb-mycTG mice (n = 6) and matching WT littermate controls (n = 6) at the age of 6–8 weeks were repetitively (twice a week) treated with CCl4 for 2 weeks and sacrificed 48 h after the last injection. (A–F) Analysis of fibrosis markers (A) Sirius red staining of liver paraffin sections showing accelerated onset of fiber formation in Alb-mycTG mice. (B) Quantification of Sirius Red-stained tissue area using image analysis software. (C) Quantification of hepatic α-SMA mRNA expression. (D) Protein expression of α-SMA, PCNA and collagen I. (E) Ki-67 immunostaining of paraffin sections showing increased cell proliferation (brown) of hepatocytes in Alb-mycTG liver. (F) Quantification of Ki-67 positive cells shown in percentage of Ki-67 positive hepatocytes and non-parenchymal cells (NPC) per high power field. (G) Determination of hepatic c-myc mRNA expression after 2 weeks of CCl4 treatment. (H) Protein expression analysis of c-myc. *: p < 0.05; **: p < 0.01; ***: p < 0.001.
specific effects in this setting at least within a time period of ten days. However, we cannot exclude that stress signals during cell isolation in the in vitro experiments overlapped with c-myc dependent paracrine mechanisms. It has also been considered that c-myc/PDGF-B-mediated liver fibrogenesis may require long latency which is best illustrated by the age-dependent increase of PDGF-B-positive hepatocytes in alb-myc\(^{\ddagger}\) mice. We therefore hypothesize that persistent physical interaction of c-myc expressing hepatocytes with HSC in vivo is necessary to explain HSC pre-activation and enhanced hepatic collagen deposition in alb-myc\(^{\ddagger}\) mice. In addition, this pro-fibrotic tissue environment may facilitate enhanced fibrosis induced by a second hit such as CCL\(_4\).

Our conclusions and key findings are summarized in Fig. 8: Up-regulation of c-myc in hepatocytes either due to genetic gene amplification or as a response to inflammatory liver injury results in moderate hepatocyte apoptosis, enhanced hepatocyte proliferation and aberrant expression of PDGF-B. Close physical vicinity of dying or PDGF-expressing hepatocytes with resident quiescent HSC can synergistically lead to HSC pre-activation and moderate transdifferentiation into myofibroblasts with subsequent mild collagen production. These primed HSC have a higher potential to produce extracellular matrix especially after a second pro-fibrotic hit. Therefore, overexpression of c-myc is a novel marker of liver fibrosis in man and mice which could be useful for improved diagnosis.

**Disclosure**

All authors declare that they have no competing interests.

**Acknowledgements**

This work was supported by the Deutsche Forschungsgemeinschaft (DFG), SFB/TRR57, project P04 (to C.L. and C.T) and by the START program of the Faculty of Medicine, RWTH Aachen to Y.A.N. We are grateful for the excellent assistance of Sibille Sauer-Lehnen, Carmen C. Tag and the Core Unit “Q3-Cell Isolation” of the SFB/TRR57 with the isolation process.
of primary HSCs. We also would like to thank Snorri S. Thorgeirsson for providing all-myc<sup>fl</sup> mice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbadis.2013.06.001.

References