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STAT5 deficiency in hepatocytes reduces diethylnitrosamine-induced liver tumorigenesis in mice

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ABSTRACT

Chronic liver diseases and the development of hepatocellular carcinoma are closely linked and pose a major medical challenge as treatment options are limited. Animal studies have shown that genetic deletion of the signal transducer and activator of transcription (STAT) 5 in liver is associated with higher susceptibility to fatty liver disease, fibrosis and cancer, indicating a protective role of hepatic STAT5 in mouse models of chronic liver disease. To investigate the role of STAT5 in the etiology of liver cancer in more detail, we applied the chemical carcinogen diethylnitrosamine (DEN) to mice harboring a hepatocyte-specific deletion of *Stat5* (S5KO). At 8 months after DEN injections, tumor formation in S5KO was significantly reduced. This was associated with diminished tumor frequency and less aggressive liver cancer progression. Apoptosis and inflammation markers were not changed in S5KO livers suggesting that the reduced tumor burden was not due to impaired inflammatory response. Despite reduced mRNA expression of the DEN bio-activator cytochrome P450 2e1 (*Cyp2e1*) in S5KO livers, protein levels were similar. Yet, delayed tumor formation in S5KO mice coincided with decreased activation of c-Jun N-terminal Kinase (JNK). Taken together, while STAT5 has a protective role in fatty liver-associated liver cancer, it exerts oncogenic functions in DEN-induced liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer-related death worldwide and accounts for ~90% of primary liver cancers [1–4]. Typically, HCC arises from chronic viral infections (HCV and HBV), alcohol-induced liver injuries, or from obesity- and type 2 diabetes mellitus-induced non-alcoholic fatty liver diseases (NAFLD) [5–7]. HCC progression is a long-lasting stepwise process in which the liver is constantly exposed to injury, such as metabolic/oxidative stress and inflammation. Chronic damage to the liver

Non-standard abbreviations: STAT, signal transducer and activator of transcription; DEN, diethylnitrosamine; CYP, cytochrome P450; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; GH, growth hormone; GHR, growth hormone receptor; JAK, Janus kinase; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; H&E, haematoxylin and eosin; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; Tnfα, tumor necrosis factor alpha; Il, interleukin; Emr1, EGF-like module-containing mucin-like hormone receptor-like 1; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; p21, p21CIP1/WAF1

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ultimately results in liver cirrhosis that might further transform, under the influence of clonal selection, into HCC [8]. Due to its heterogeneity and complexity, treatment options are limited and HCC is therefore associated with a poor survival prognosis [9]. Thus, there is an urgent need to better understand the mechanisms that contribute to the pathogenesis of HCC.

Growth hormone (GH) regulates whole body physiology to execute dynamic cellular processes [10–12]. In hepatocytes, GH binding to its receptor (GHR) activates Janus kinase (JAK) 2, which in turn activates signal transducer and activator of transcription (STAT) 5A and STAT5B (collectively referred to as STAT5). Activated STAT5 translocates to the nucleus to regulate genes involved in vital liver functions, including postnatal body growth, cell cycle progression, lipid, bile acid, reactive oxygen species (ROS) and drug metabolism [12–18]. Even though STAT1 and STAT3 can be activated by GH in cases of diminished STAT5 expression [19], STAT5 is the principal mediator of physiologic GH signaling [12,20]. In addition to the activation of the JAK-STAT pathway, binding of GH to the GHR activates phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways [12].

It was shown that impaired GH-STAT5 signaling correlates with chronic liver disease, i.e. the development of fatty livers is driven by loss of hepatic STAT5 or JAK2 expression changing ROS, glucose and lipid metabolism [18,21–26]. In addition, STAT5 deficiency in mice has been associated with higher susceptibility to liver fibrosis and cancer, suggesting that hepatic STAT5 has a protective role in mouse models of chronic liver disease by preventing chronic liver damage and tumorigenesis [18,27–29]. Although the JAK2-STAT5 pathway was prominently explored for liver cancer etiology, the role of STAT5 in chemical-induced tumorigenesis remains enigmatic.

In the present study, we aimed to investigate the impact of hepatic STAT5 on liver tumorigenesis by using the diethylnitrosamine (DEN)-induced liver cancer model, at which DEN initiates the development of liver tumors by alkylating DNA. To address STAT5 function in DEN-induced tumorigenesis, we used animals with hepatocyte-specific *Stat5* deletion (S5KO [30], using *Alfp-cre* [31]).

2. Materials and methods

2.1. Animal experiments

Hepatocyte- and cholangiocyte-specific STAT5-deficient mice (S5KO; C57BL/6 x Sv/129) were generated by crossing *Stat5ab floxed* [30] with *Alfp-cre* transgenic mice [31]. *Alfp-cre* negative littermates served as controls. Animals were housed under standardized conditions (12 h dark/12 h light cycle) and had free access to water and a regular rodent diet (Ssniff EF, R/M Kontrolle, Ssniff GmbH, Germany). Animal studies were approved by the Austrian government and the Medical University of Vienna (BMWF-66.009/0139-C/GT/2007, BMWF-66.009/0092-II/10b/2009). For all experiments, male mice were used. Mice were injected intraperitoneally with 25 mg/kg of DEN (Sigma-Aldrich, Munich, Germany) at 14 days of age and euthanized via CO_2 inhalation 2 months, 8 months, or 10 months after DEN injection. No premature mortality was observed in DEN-treated mice of either genotype.

2.2. Plasma biochemistry

To determine plasma concentrations of biochemical parameters, blood of euthanized mice was harvested by heart puncture. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using the test strip-based Reflotron Plus analyzer (Roche, Basel, Switzerland).

2.3. Histology and immunohistochemistry

Livers were fixed in 4% formaldehyde, dehydrated, paraffin-embedded, sliced, and stained with haematoxylin and eosin (H&E) using standard procedures and analyzed by light microscopy. A blinded evaluation of liver histology was performed by a board-certified pathologist (JH). Evaluation of lobular inflammation, apoptosis and steatosis were similarly assessed by the NAFLD activity scoring (NAS) system for human liver biopsies using H&E stained sections [32]. Ki-67, cleaved Caspase 3 and pH2AX staining was performed as previously described [27,29]. Quantification of positive cells was achieved using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997–2016.).

2.4. Molecular analyses

RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany). 1 μg of RNA was reverse transcribed (Revert Aid cDNA synthesis kit; Thermo Fisher Scientific, Waltham, MA, USA) and quantitative real-time PCR (qPCR) was performed on the CFX96 Real-Time System (BioRad, Hercules, CA, USA) using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA). Each reaction condition was performed in duplicate. Gene expression was normalized to *Gapdh* mRNA. Relative abundance was calculated using the $2^{-\Delta Ct}$ method (gene-specific expression level relative to that of the reference gene). Primer sequences are provided in Supplementary Table 1.

Western blot analyses (100 μg protein) were performed using standard techniques. Primary antibodies against pY694-STAT5 (#71-6900, Invitrogen, Camarillo, CA, USA), pY705-STAT3 (#9131), pSer473-Akt (#9271), pT202/Y204-ERK1/2, pT183/Y185-SAPK/JNK, pT180/Y182-p38 MAPK (Phospho-MAPK Family Antibody Sampler Kit #9910), ERK1/2, SAPK/JNK, p38 (MAPK Family Antibody Sampler Kit #9926; all from Cell Signaling, Danvers, MA, USA), STAT5 (sc-835), STAT3 (sc-7179), Akt (sc-8312), HSC70 (sc-7298; all from Santa Cruz Biotechnology, CA, USA) were used. The intensity of protein bands was quantified by densitometry using the ImageJ software (NIH, Bethesda, MD, USA).

2.5. Statistical analyses

Statistics were performed with GraphPad Prism® (La Jolla, CA, USA). Data are presented as mean ± SEM. Statistical analyses were preformed comparing the two experimental groups within one time point with either two-tailed Student's *t*-test or Wilcoxon rank-sum test using a confidence interval of 95%. Differences between the groups were considered statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Delayed liver tumorigenesis in S5KO mice

We and others have shown that hepatic STAT5 deficiency promotes chronic liver damage and tumorigenesis [18,27–29]. To determine how a hepatocyte specific deletion of STAT5 affects liver tumor initiation and progression in a model of chemical induced tumorigenesis, we exposed mice to DEN, a well-established carcinogen in experimental models. Mice were injected with a single dose of DEN at 14 days of age and euthanized 2, 8 or 10 months after injection (Fig. 1A). Analysis of liver damage parameters revealed that ALT levels were increased in S5KO mice at 2 and 8 months after DEN injection, but lower ALT levels were seen compared to controls at 10 months after injection (Fig. 1B). No drastic changes were observed in AST levels at 2 and 10 month after DEN injection between the genotypes; only at 8 months after injection AST levels were increased in S5KO mice (Fig. 1B). Liver to body weight ratios were increased in S5KO mice at 2 month of age (Fig. 1C). This

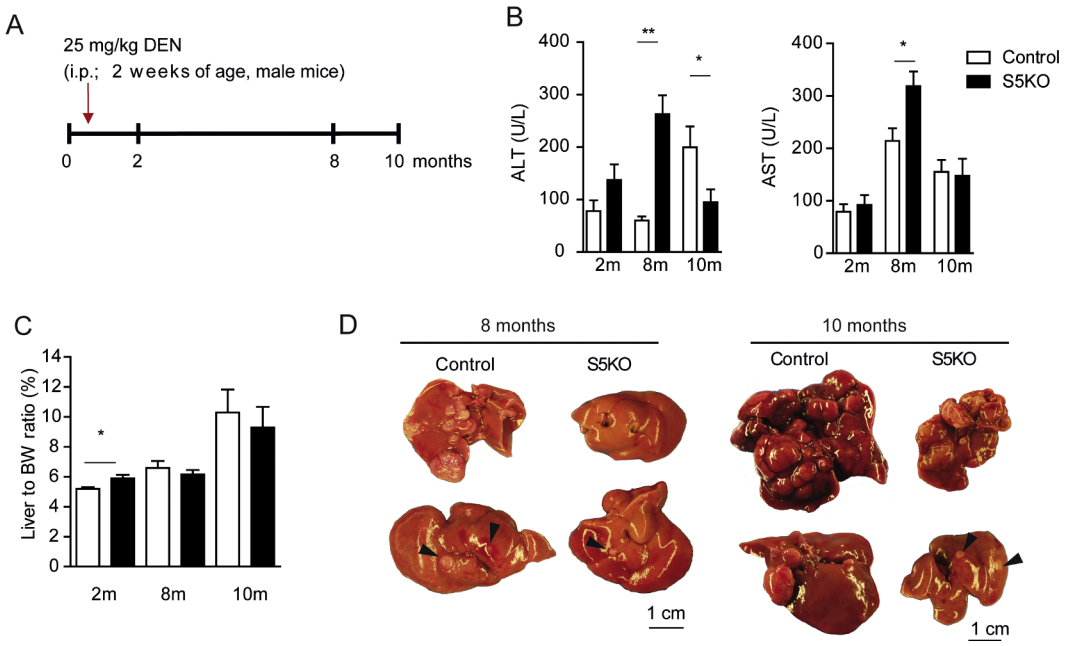

Fig. 1. Assessment of liver damage parameters and macroscopic appearance of livers after DEN injection. (A) Scheme of the experimental setup. Mice were intraperitoneally injected with DEN (25 mg/kg) at 2 weeks of age and tissue was harvested at 2, 8 and 10 months after injection. (B) Plasma levels of ALT and AST at indicated time points after DEN injection (n ≥ 6). (C) Liver to body weight (BW) ratio at indicated time points after DEN injection (n ≥ 6). (D) Macroscopic appearance of tumorigenic livers at indicated time points after DEN injection. *p < 0.05, **p < 0.01.

was due to the pronounced hepatic steatosis that is prominently developing upon STAT5 loss [27]. However, significant changes in liver weights were no longer observed at 8 and 10 months of DEN injection (Fig. 1C). Macroscopic appearance of S5KO livers suggested a less pronounced response to DEN induced liver tumorigenesis at the final stage of analysis in 10 months old mice (Fig. 1D) fitting to the normalization of liver to body weight ratios at older age.

Histological analysis confirmed a reduced tumor incidence in S5KO mice 8 months after DEN injection: at this time point 100% of control mice developed tumors but only one third of S5KO mice displayed tumor nodules (Fig. 2A and B). By 10 months after DEN injection, tumor incidence was 100% in both genotypes. However, tumor frequency in S5KO mice was significantly lower at 8 months and slightly reduced at 10 months after DEN treatment (Fig. 2C). 8 months after treatment, tumors that developed in S5KO mice were characterized as solid tumors, while in addition to a solid growth pattern in control mice, 20% of tumors displayed a mixed tumor appearance (Fig. 2D). 10 months after treatment, control mice showed predominantly solid tumors (72%), indicative for aggressive growth, but also trabecular (6%) or mixed (22%) growth patterns (Fig. 2D). By contrast, only 23% of STAT5-deficient tumors were characterized by a solid growth pattern and 77% displayed a mixed growth pattern suggesting tumors of S5KO were less aggressive compared to tumors of control mice. As assessed by Ki-67 staining, there was no drastic difference in the proliferation level between tumors of control and S5KO mice (Fig. 2E and F).

Together, these results demonstrate that hepatic STAT5-deficiency delays tumor formation in a DEN-induced model of liver tumorigenesis and reduces tumor frequency with considerably less aggressive tumor growth 10 months after DEN treatment.

3.2. Reduced tumor burden in S5KO mice is not linked to changes in apoptosis and inflammation

Histological assessment revealed there were no drastic differences in lobular inflammation and apoptosis between the genotypes at 2, 8 or 10 months after DEN injection (Fig. 3A, B, Suppl. Fig.S1A, 1B and Suppl. Fig. 2A, 2B). As expected, steatosis was more pronounced in S5KO livers (Fig. 3C and Suppl. Fig. 1C). Next, we evaluated the gene expression of inflammatory markers such as tumor necrosis factor alpha (*Tnfa*), interleukin 6 (*Il6*), interleukin 1 beta (*Il1b*) and interleukin 10 (*Il10*) (Fig. 3D). No major differences were observed in mRNA expression levels of *Il6* and *Il1b*. Although not statistically significant, *Tnfa* mRNA levels were increased in S5KO livers at 10 months after DEN injection and *Il10* mRNA expression was increased in S5KO livers at 2 and 8 months after DEN injection. In addition, no significant differences were observed in the expression of the major macrophage marker EGF-like module-containing mucin-like hormone receptor-like 1 (*Emr1)* which is coding for F4/80 (Suppl. Fig. 2C).

These data suggest that reduced tumor burden in STAT5-deficient animals is not a consequence of prominent changes in inflammation or apoptosis.

3.3. Hepatic STAT5-deficiency reduces JNK activation

To investigate whether STAT5-deficiency alters DEN metabolism, we analyzed the expression of a cytochrome P450 (CYP) protein, which was shown to be essential for catalyzing DEN activation in the liver. Interestingly, mRNA levels of *Cyp2e1*, which represents the major CYP that is required for DEN bio-activation [33], were lower in livers of S5KO mice compared to controls at 2, 8 and 10 months after DEN injection (Fig. 4A). However, Western blot analysis revealed that the decreased *Cyp2e1* mRNA expression did not translate into diminished CYP2E1 protein expression. CYP2E1 was similarly expressed on protein level in both genotypes at 2 months and 8 months after DEN treatment, while it was increased in S5KO livers at 10 months after treatment (Fig. 4B).

To further gain insight into molecular changes that underlie the differences in tumorigenesis between control and S5KO mice, we determined the activation of major stress-dependent MAPK signaling pathways. These are induced by liver damage and are known to be involved in the pathogenesis of liver tumor development and progression. We observed slightly decreased c-Jun N-terminal kinase (JNK) and P38 activation in S5KO livers at 2 months of age (Suppl. Fig. 3A). There were no drastic differences observed in the activation of extracellular

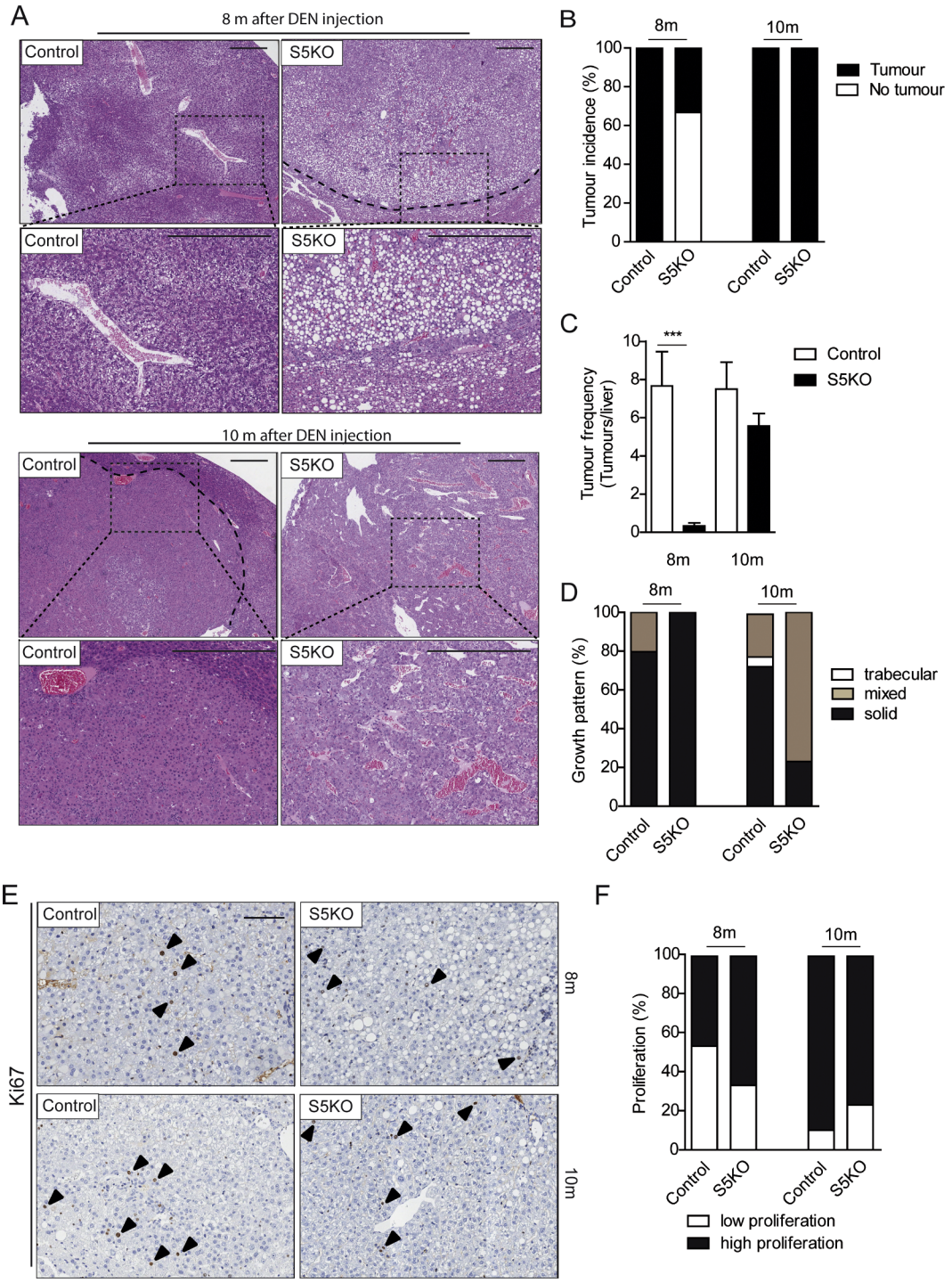

Fig. 2. Loss of STAT5 leads to delayed tumor formation and frequency with less aggressive tumors after DEN injection. (A) Representative H&E staining of liver tumor sections at indicated time points after DEN injection. The scale bar indicates 500 μm and bold dashed lines surround the tumor tissue. (B) Based on macroscopic tumor nodules, tumor incidence of livers was analyzed at indicated time points after DEN injection. (C) Tumor frequency of livers at indicated time points after DEN injection was analyzed based on macroscopic tumor nodules ($n \geq 6$). (D) Histopathological analysis assessing the tumor growth pattern. (E) Representative Ki-67 staining of liver sections at indicated time points after DEN injection and (F) histopathological analysis. The scale bar indicates 100 μm. $^{***}p < 0.001$.

signal-regulated kinases (ERK) 1/2 and P38 between control and S5KO livers at 2, 8 or 10 months after DEN injection (Fig. 5A, Suppl. Fig. 3A and B). Although no significant changes in JNK activation were observed after 8 months of DEN treatment (Suppl. Fig. 3B), S5KO mice displayed significantly reduced JNK activity at 10 months after DEN injection (Fig. 5A). Although DNA damage, as assessed by pH2AX staining, was slightly increased in S5KO livers at 2 months of age, we found a reduced pH2AX staining in S5KO livers at 8 and 10 months of age (Fig. 5B and C). No significant differences were observed in serine 473 phosphorylation of Akt and activation of STAT3 was not significantly changed in S5KO livers (Fig. 5A, Suppl. Fig. 3A and 3B). As expected, STAT5 was activated in control livers and not significantly detectable in S5KO mice (Fig. 5A, Suppl. Fig. 3A and 3B).

In summary, decreased tumor incidence and tumor frequency with less aggressive tumors observed in S5KO mice coincides with decreased activation of JNK. However, CYP2E1 protein levels and inflammation

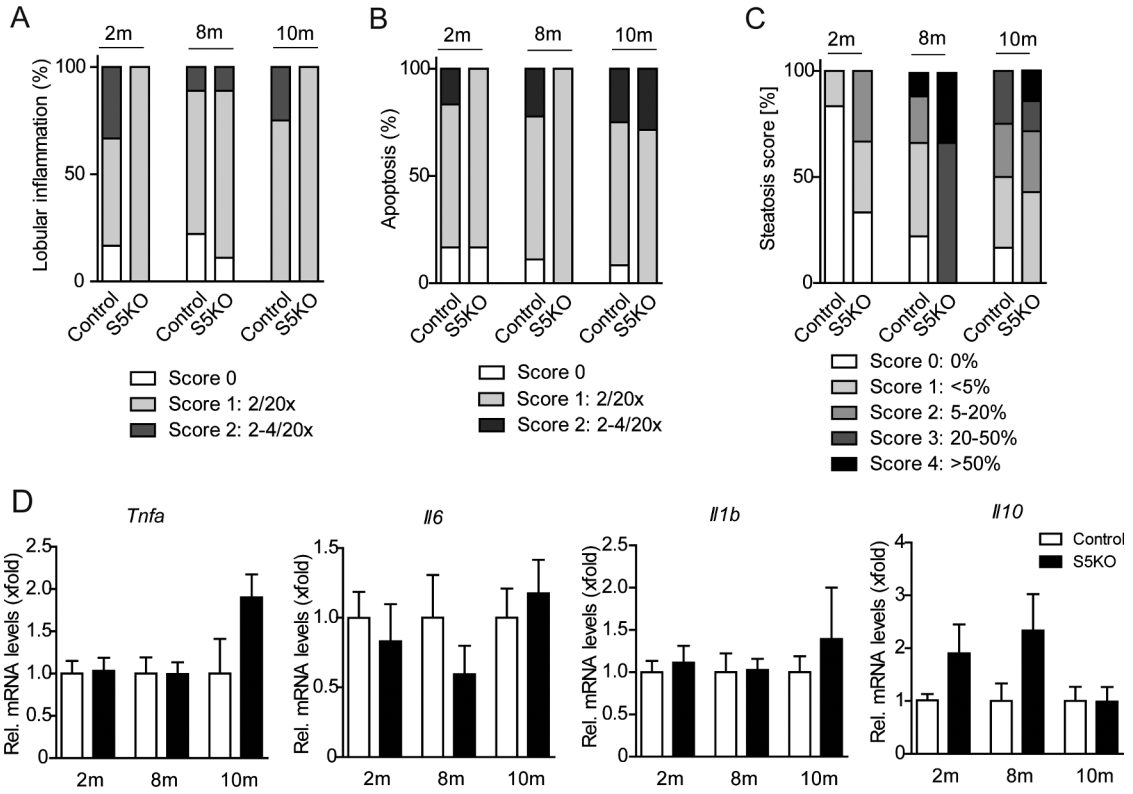

Fig. 3. Characterization of liver histopathology reveals no differences in inflammation and apoptosis. (A–C) Histopathological analysis assessing lobular inflammation (A), apoptosis (B) and steatosis (C) of liver sections. (D) mRNA levels of inflammatory marker genes. Ct values were normalized to *Gapdh* (n ≥ 5).

markers were unchanged suggesting that tumorigenesis was not driven by inflammatory events.

4. Discussion

In this study, we report that STAT5-deficiency in hepatocytes delays liver tumorigenesis in a model of chemically-induced carcinogenesis with DEN.

It has been shown that STAT5 activity is often implicated in malignant diseases with poor prognosis [34], such as hematopoietic and prostate cancers [35–38] portraying STAT5 as an oncogene. However, STAT5 activation has also been associated with a more favorable prognosis in breast cancer patients for tumor initiation [39,40] indicating that the role of STAT5 in cancers is more complex, especially when it comes to cell-type specific functions of carcinoma formation and progression. STAT5 function in HCC is barely defined. Yet, as HCC is the 3rd leading cause of cancer-related deaths [2], it is important for HCC etiology to define key proteins involved in HCC development. So far, aberrant activation of STAT5 was reported in patients with HBV-related HCCs [41,42]. However, animal studies demonstrated that loss of STAT5 activity is associated with higher susceptibility to liver fibrosis and cancer, suggesting that hepatic STAT5 has a protective role in mouse models of chronic liver disease [18,27–29]. Our results show that tumor incidence, frequency and aggressiveness is reduced in mice lacking hepatic STAT5 suggesting that STAT5 exerts oncogenic functions in DEN-induced liver cancer. In support of our findings recently published data showed delayed tumor formation in mice harboring a hepatic deletion of JAK2, the prototypical upstream kinase of STAT5, when treated with DEN [43]. In accordance with our data, it was shown in JAK2 deficient mice that activation of stress-activated protein kinase JNK is diminished compared to controls. Consistently, it has been reported that JNK deficiency prevents DEN-induced liver cancer in mice [44] and that hepatic deficiency of cJun – a downstream target of JNK – results in reduced liver tumors upon DEN treatment [45]. The importance of aberrant activation of JNK and cJun is also supported by mouse models of chronic liver disease, which show increased JNK and cJun activity in STAT5-deficient mice, thereby enhancing liver tumorigenesis [27,29]. Nevertheless, another explanation for reduced JNK

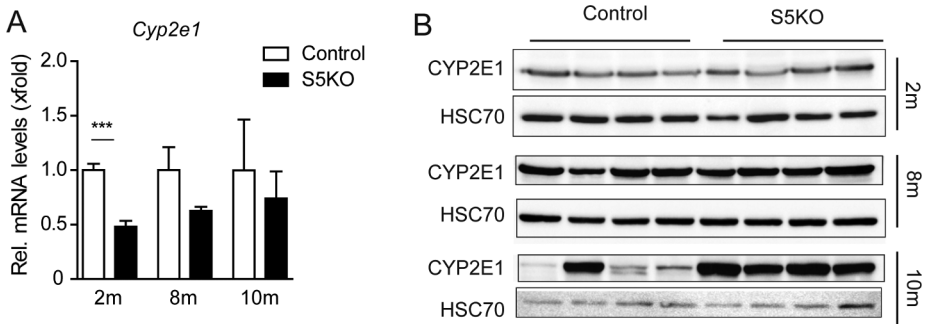

Fig. 4. Reduced *Cyp2e1* mRNA levels in STAT5-deficient livers. (A) mRNA levels of *Cyp2e1*. Ct values were normalized to *Gapdh* (n ≥ 5). $^{***}p < 0.001$. (B) Protein expression of CYP2E1 in liver lysates at indicated time points after DEN injection. HSC70 is shown as loading control (n = 4).

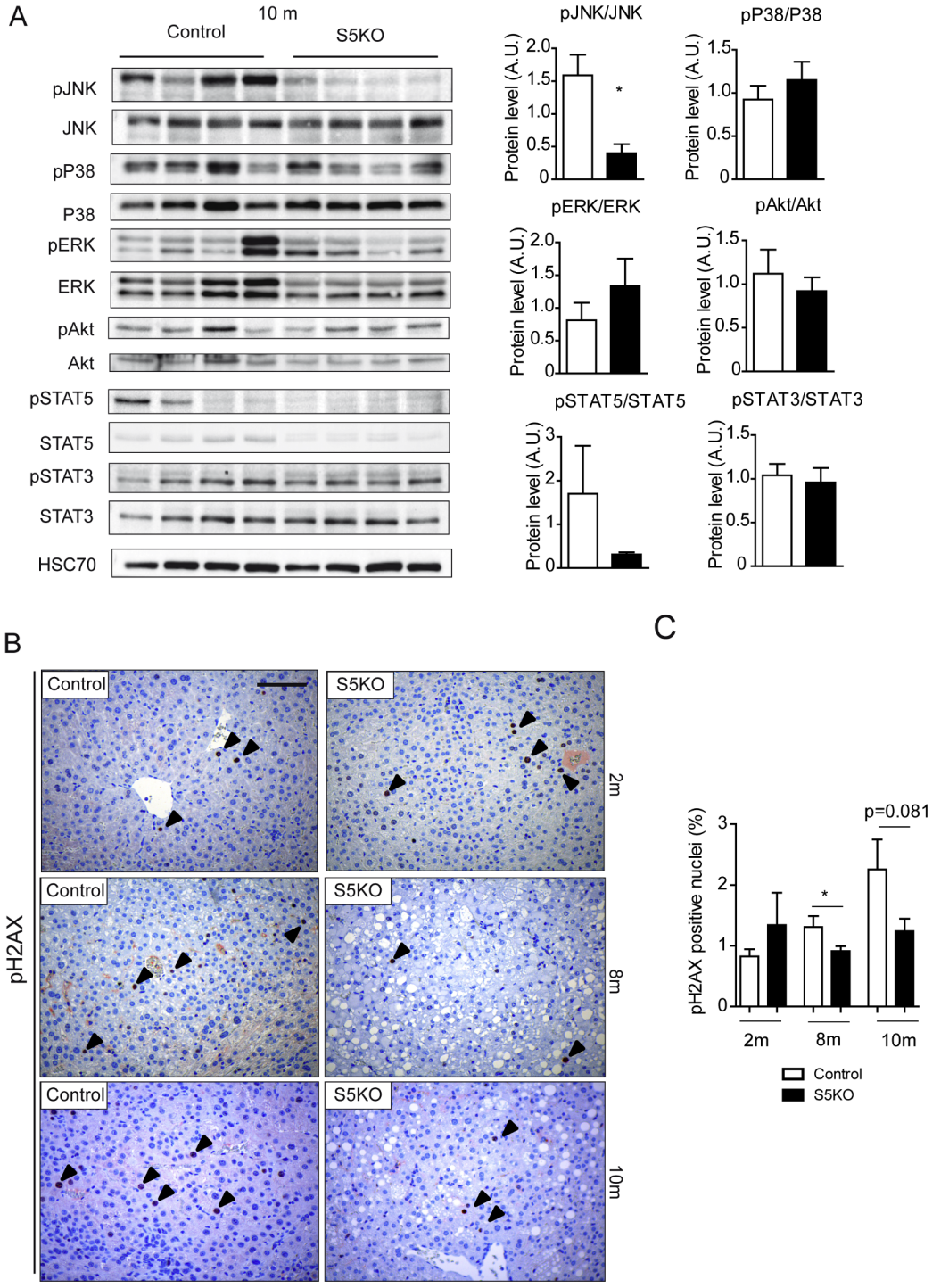

Fig. 5. Analysis of oncogenic signaling pathways reveals reduced activation of JNK in STAT5-deficient mice. (A) Western blot analysis of oncogenic signaling pathways in whole liver homogenates 10 months post DEN injection. A representative HSC70 is shown as loading control (n = 4). (B) Representative liver sections stained with antibodies against pH2AX to detect DNA damage at indicated time points after DEN injection and (C) quantification of positive staining was performed using ImageJ (n ≥ 5). The scale bar indicates 100 μm. *p < 0.05.

activity seen in S5KO mice at 10 months after DEN injection could be attributed to lower tumor tissue rather than a cause of less induced JNK signaling. Similarly, changed protein levels of the DEN bio-activator CYP2E1 were only found in S5KO mice at 10 months after DEN injection. However, protein levels of CYP2E1 in less transformed S5KO livers might be relatively increased when compared to more transformed hepatocytes in control livers, which might generally express less CYP2E1. To further address the importance of differences seen in JNK activity and CYP2E1 protein expression, assessment at time points shortly after DEN injection (24–72 h) might be more informative. Future studies focusing on short-term responses to DEN administration with DNA damage response pathway monitoring may provide valuable insights into mechanisms that underlie delayed tumor initiation in S5KO mice upon chemical mutagenesis.

Hepatic STAT5 deficiency is linked to compensatory activation of STAT3, which accelerates tumorigenesis in mice [23,27,29]. Yet, in our study no significant differences in aberrant STAT3 activation were observed between DEN-treated control and STAT5-deficient mice. This is

not in line with DEN-induced tumorigenesis in JAK2-deficient mice, where STAT3 activation was significantly increased compared to control mice [43]. Likewise, while signal transduction of Akt, p38 and ERK signaling, does not account for the differences in tumorigenesis seen in our study, ERK activity was increased in JAK2-deficient mice when treated with DEN [43]. Yet, the increase in STAT3 and ERK activation seemed insufficient for tumor promotion in JAK2-deficient mice upon DEN treatment.

In mice, hepatic STAT5 deficiency has been linked to abolished p53 activity that translated into increased DNA damage, thereby contributing to early liver tumorigenesis [29]. In line, it has been reported that STAT5 induces several downstream targets of p53 in mouse embryonic fibroblasts, which was reversed upon STAT5 deficiency [18]. These results may suggest a regulatory role of STAT5 in the control of p53 function in liver. This is in contrast to our data, where hepatic deletion of STAT5 led to reduced DNA damage at 8 and 10 months. This suggests that p53 activation and DNA damage response was intact in S5KO mice after DEN injection. Furthermore, RAD51, which is essential for the repair of DNA double-strand breaks by homologous recombination, has been shown to be overexpressed in cancers and has been linked to increased resistance to cancer therapies and increased invasiveness [46–48]. Normally, RAD51 is inhibited by p53 [49], but it has been shown that its expression is mediated by STAT5 in leukemic cancers associated with higher ROS metabolism [48]. Linking this finding to our study would suggest that RAD51 expression in our S5KO should be low, which in part would explain why tumor onset is delayed in S5KO. However, to confirm this hypothetical role in regard to RAD51 regulation further experiments on DNA damage response pathways need to be undertaken.

In our study, we demonstrated that the role of hepatic STAT5 seems to be context-specific, as STAT5 exerts oncogenic functions in DEN-induced liver cancer, but tumor suppressive functions in mouse models of chronic liver disease. Context specificity was also reported for p21CIP1/WAF1 protein (p21), as it was first considered to be a classical tumor suppressor [50], but later linked to oncogenic functions as well [51,52]. Elevated levels of p21 were shown to be present in many different cancers correlating with poor prognosis [51,53,54]. This was further confirmed when elevated levels of p21 were observed in livers of SOCS1-deficient mice treated with DEN, which showed increased tumor burden. These findings indicated that p21 might have an oncogenic role in liver tumorigenesis [55]. Interestingly, loss of STAT5 leads to diminished p21 expression in mouse embryonic fibroblasts and mice [16] suggesting that a decrease of oncogenic p21 might delay tumorigenesis in our model. Although diminished p21 levels in STAT5-deficient mice was reported to enhance hepatocyte proliferation upon CCL_4 treatment [16], which would rather contribute to liver tumorigenesis, p21 function might be different in DEN-induced liver cancer. This argues that the complete spectrum of p21 functions during tumorigenesis, similar to STAT5, is mutational-context dependent.

Overall, previous studies suggest that hepatic STAT5 has a protective role in mouse models of chronic liver disease by preventing chronic liver damage and tumorigenesis. Yet, it promotes tumorigenesis in a DEN-induced liver cancer model. As a result, the role of STAT5 in liver cancer appears to depend on environmental and genetic factors, and cell-type specific settings, as it is similarly reported with other STAT protein family members [56–62]. Therefore, to get further insights and to understand the explicit role of STAT5 in the heterogeneous etiology of liver cancer, it is critical to study different liver cancer models in more depths.

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Duality of interest

The authors have no potential conflict of interest to report.

Author contribution

DK, MT and KMM designed the experiments; and RM supervised the study; DK, MT and KS performed most of the experiments, with contributions from KMM and KF; DK performed most of the data analyses, with contributions from MT, KMM and NGS; JH performed histopathologic analysis; DK, MT, KMM, JH and RM interpreted the data; DK, MT and RM wrote the manuscript; KMM edited the manuscript; LK provided intellectual and histopathology input; and KMM, KS, NGS, KF, LK, JH and RM critically revised the manuscript. All authors approved the final version of the manuscript. RM is responsible for the integrity of the work as a whole.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.10.014.

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