Loss of c-Jun N-terminal Kinase 1 and 2 Function in Liver Epithelial Cells Triggers Biliary Hyperproliferation Resembling Cholangiocarcinoma

Francisco Javier Cubero, Mohamed Ramadan Mohamed, Marius M. Woitok, Gang Zhao, Maximilian Hatting, Yulia A. Nevzorova, Chaobo Chen, Johannes Haybaeck, Alain de Bruin, Matias A. Avila, Mark V. Boekschoten, Roger J. Davis, and Christian Trautwein

Targeted inhibition of the c-Jun N-terminal kinases (JNKs) has shown therapeutic potential in intrahepatic cholangiocarcinoma (CCA)-related tumorigenesis. However, the cell-type-specific role and mechanisms triggered by JNK in liver parenchymal cells during CCA remain largely unknown. Here, we aimed to investigate the relevance of JNK1 and JNK2 function in hepatocytes in two different models of experimental carcinogenesis, the diethylnitrosamine (DEN) model and in nuclear factor kappa B essential modulator (NEMO) hepatocyte-specific knockout (Δhepa) mice, focusing on liver damage, cell death, compensatory proliferation, fibrogenesis, and tumor development. Moreover, regulation of essential genes was assessed by reverse transcription polymerase chain reaction, immunoblottings, and immunostainings. Additionally, specific Jnk2 inhibition in hepatocytes of NEMO Δhepa/JNK Δhepa mice was performed using small interfering (si) RNA (siJnk2) nanodelivery. Finally, active signaling pathways were blocked using specific inhibitors. Compound deletion of Jnk1 and Jnk2 in hepatocytes diminished hepatocellular carcinoma (HCC) in both the DEN model and in NEMO Δhepa mice but in contrast caused massive proliferation of the biliary ducts. Indeed, Jnk1/2 deficiency in hepatocytes of NEMO Δhepa animals caused elevated fibrosis, increased apoptosis, increased compensatory proliferation, and elevated inflammatory cytokines expression but reduced HCC. Furthermore, siJnk2 treatment in NEMO Δhepa/JNK Δhepa mice recapitulated the phenotype of NEMO Δhepa/JNK Δhepa mice. Next, we sought to investigate the impact of molecular pathways in response to compound JNK deficiency in NEMO Δhepa mice. We found that NEMO Δhepa/JNK Δhepa livers exhibited overexpression of the interleukin-6/signal transducer and activator of transcription 3 pathway in addition to epidermal growth factor receptor (EGFR)-rapidly accelerated fibrosarcoma (Raf)-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) cascade. The functional relevance was tested by administering lapatinib, which is a dual tyrosine kinase inhibitor of erythroblastic oncogene B-2 (ErbB2) and EGFR signaling, to NEMO Δhepa/JNK Δhepa mice. Lapatinib effectively inhibited cystogenesis, improved transaminases, and effectively blocked EGFR-Raf-MEK-ERK signaling. Conclusion: We define a novel function of JNK1/2 in cholangiocyte hyperproliferation. This opens new therapeutic avenues devised to inhibit pathways of cholangiocarcinogenesis. (Hepatology Communications 2020;4:834-851).

Bile duct hyperplasia and aberrant cholangiocyte growth can result in hepatic cystogenesis, differentially diagnosed on the basis of cholangioma, cholangiofibrosis, intrahepatic cholangiocarcinoma (CCA), and oval cell hyperplasia. CCA, a malignancy that arises in the setting of chronic inflammation of biliary epithelium cells, has an increasing incidence and is the second most common primary

Abbreviations: α-SMA, alpha smooth muscle actin; Δhepa, hepatocyte-specific knockout; Ab, Abet-N1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; CAI, cancer-associated fibroblast; CC3, cleaved caspase 3; CCA, cholangiocarcinoma; CK, creatine kinase; CLD, chronic liver disease; Col1A1, collagen type I alpha 1; DEN, diethylnitrosamine; dmbt1, deleted in malignant brain tumors 1; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; ERBB, erythroblastic oncogene B; ERK, extracellular signal-regulated kinase; f/f, floxed mice; gabrbp, gamma-aminobutyric acid A receptor, pi; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HNF, hepatocyte nuclear factor; HPF, high-power field; IHC, immunohistochemistry; IL, interleukin; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LoxP, locus of X-over P1; LPC, liver parenchymal cell; LW, liver weight; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; mRNA, messenger
liver cancer globally. Unfortunately, survival beyond a year of diagnosis is less than 5% and therapeutic options are scarce.(3) Several in vivo and in vitro models as well as research with human tissue samples help to elucidate the main pathways implicated in CCA formation. However, none of these studies recapitulates the human disease, and translation into improved patient outcome has not been achieved. In addition, the pathophysiology of CCA remains poorly understood. Thus, there is an urgent need for new models to improve the management of this insidious and devastating disease.

RNA; MUC, mucin; NEMO, nuclear factor kappa B essential modulator; NF-κB, nuclear factor kappa B; OSM, oncostatin M; p, phosphorylated; PCNA, proliferating cell nuclear antigen; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; Raf, rapidly accelerated fibrosarcoma; RIPK, receptor-interacting serine/threonine-protein kinase; si, small interfering; SOCS3, suppressor of cytokine signaling 3; SOX-9, transcription factor SOX9; STAT, signal transducer and activator of transcription; TKI, tyrosine kinase inhibitor; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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*These authors contributed equally to this work.

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The c-Jun N-terminal kinases (JNKs) are evolutionarily conserved mitogen-activated protein kinases (MAPKs) and play an important role in converting extracellular stimuli into a wide range of cellular responses, including inflammatory response, stress response, differentiation, and survival. In tumorigenesis, JNK has been shown to have tumor suppressive function in breast, prostate, lung, and pancreas cancer. However, the pro-oncogenic role for JNK has also been well documented. Importantly, JNK has lineage-determinant functions in liver parenchymal cells (LPCs) where it not only favors proliferation of biliary cells but also directly biases biliary cell-fate decisions in bipotential hepatic cells. It has been reported that JNK inhibition delays CCA progression by impeding JNK-mediating biliary proliferation. These data indicate that JNK modulation would be of therapeutic benefit in patients with CCA. Nevertheless, little is known about the cell-type-specific role and mechanism of JNK in biliary overgrowth in order to have a targeted and definite therapy against CCA.

In the present study, we investigated the implications of hepatocyte-defective JNK signaling in experimental carcinogenesis. Unexpectedly, loss of Jnk1/2 in LPCs inhibited hepatocellular carcinoma (HCC) but triggered biliary epithelium hyperproliferation and features compatible with CCA. Overall, our data uniformly suggest that hepatocytic JNK is pivotal for biliary epithelial hyperproliferation resulting in ducto/cystogenesis.

Materials and Methods

GENERATION OF MICE AND ANIMAL EXPERIMENTS

Albumin (Alb)-Cre and Jnk2-deficient mice in a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME). Jnk1 locus of X-over P1(LoxP/LoxP)/Jnk2Δ−/− (Hepatocyte-specific knockout of Jnk1 [JNK1Δhepa]) mice were created as reported. We used male mice for all experiments. For in vivo experiments, mice were treated with a daily dose of lapatinib (150 mg/kg body weight; n = 7 mice per group) or vehicle (0.5% hydroxypropylmethylcellulose/1% Tween 80) (n = 6) by oral gavage starting at 6 weeks of age over a period of 6 weeks. For small interfering (si)RNA-mediated knockdown experiments, 8-week-old nuclear factor kappa B (NF-κB) essential modulator (NEMO)Δhepa/JNK1Δhepa were injected with a dose of 0.2 mg/kg body weight (BW) siJnk2 or small interfering luciferase (siLuc) once per week over a period of 4 weeks. In parallel, lapatinib was given orally to siJnk2-treated NEMOΔhepa/JNK1Δhepa mice on the same day of the first siJnk2- injection. Induction of tumorigenesis was performed by intraperitoneal injection of 25 mg/kg BW of diethylnitrosamine (DEN; Sigma-Aldrich, Munich, Germany) at 14 days of age. Mice were killed 24 weeks later. Vehicle-injected (saline) male mice served as controls.

Animal experiments were carried out according to the German legal requirements and animal protection law and approved by the authority for environment conservation and consumer protection of the state of North Rhine-Westfalia (LANUV; Germany). All strains were crossed on a C57BL/6 background. The mice were housed in the Institute of Laboratory Animal Science at the University Hospital RWTH-Aachen University according to German legal requirements (Animal Welfare Act [DeutschesTierschutzgesetz], Federation of European Laboratory Animal Science Associations [FELASA], Society of Laboratory Animal Science [GV-SOLAS]) under a permit of the Veterinäramt der Städteregion Aachen. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Animal Models. All organ explants and animal experiments were approved by the local authority for environment conservation and consumer protection of LANUV on the following animal grants: 30034G (AZ-84-02.04.2016.A080) and TVA-11324GZ (AZ-84-02.04.2016.A490).

INTERFERENCE RNA AGAINST Jnk2 (siJnk2)

The siRNA molecules were purchased from Axolabs GmbH (Kulmbach, Germany) and were chosen due to their ability to specifically target Jnk2 in mice with mismatches to Jnk1 (2–18 nucleotides) to increase in vivo stability and suppression of the immune-stimulatory properties, as described.

DATA AND SOFTWARE AVAILABILITY

Affymetrix Microarray was performed as described, and data were deposited with the National Center for Biotechnology Information Gene
Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE140498.

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM. Statistical significance was determined by two-way analysis of variance (ANOVA) followed by a Student t test or by one-way ANOVA followed by a Newman-Keuls multicomparison test. P < 0.05 was considered significant.

Results

COMBINED LOSS OF Jnk1/2 FUNCTION IN HEPATOCYTES TRIGGERS BILIARY HYPERPROLIFERATION AND DUCTO/CYSTOGENESIS IN AN EXPERIMENTAL MODEL OF CHRONIC LIVER DISEASE

Mice lacking NEMO in LPCs spontaneously develop HCC, rendering these animals an ideal model that perfectly mimics progression of chronic liver disease (CLD) as observed in humans. We previously established that Jnk1 and Jnk2 have specific roles for the progression of NEMO-Δhepa-dependent chronic liver injury, indicating that the MAPK genes expressed in liver cells have pivotal functions in cell death and inflammation. We then found that combined activities of Jnk1 and Jnk2 specifically in hepatocytes protected against toxic liver injury (CCl₄ and acetaminophen). However, Jnk1/2 deficiency in hepatocytes increased tumor burden in an experimental model of CLD. Therefore, in the present study we aimed to investigate the definitive contribution of JNK genes to liver cancer.

For this purpose, we generated NEMOΔhepa/JNKΔhepa and their respective controls NEMOΔhepa and NEMOfloxed (f/f) mice (Supporting Fig. S1) and examined the progression of liver disease. At 13 weeks of age, histologic evaluation of NEMOΔhepa unveiled the presence of dysplastic nodules, steatohepatitis, and cell death (Supporting Fig. S2A,B). All NEMOΔhepa/JNKΔhepa livers with no external signs of nodules spontaneously showed hyperproliferation of biliary epithelium translated into hepatic ducto/cystogenesis and lymphoid aggregates surrounding these structures (Supporting Fig. S2A,B).

Development of HCC is characteristic of 1-year-old NEMOΔhepa mice, visible both macroscopically and histologically. However, most 52-week-old NEMOΔhepa/JNKΔhepa mice displayed no signs of HCC. Here, yellowish coloration of the liver parenchyma was associated with hyperproliferation of biliary epithelial cells and lymphoid cell accumulation. This was much more pronounced than at earlier stages of CLD. Interestingly, histopathological examination of these specimens in two different institutes (Utrecht and Graz) revealed that approximately 33% of 52-week-old JNKΔhepa livers presented small cysts while an increased frequency and a higher number of ductular cells were evident in the hepatic parenchyma of all NEMOΔhepa/JNKΔhepa, with atypia compatible with CCA (Fig. 1A,B; Table 1).

Moreover, combined JNK1/JNK2 deletion in hepatocytes of NEMOΔhepa mice triggered significantly reduced BW and liver weight (LW) compared with NEMOΔhepa mice but a similar LW/BW ratio as hepatocyte-specific NEMO-deficient mice (Fig. 1C). Notably, at 13 weeks of age, NEMOΔhepa/JNKΔhepa animals had a significantly increased hepatosomatic ratio compared with NEMOΔhepa mice, albeit no differences in BW or LW (Supporting Fig. S2C).

Surprisingly, 1 year-old NEMOΔhepa/JNKΔhepa livers exhibited reduced HCC compared with NEMOΔhepa animals (Fig. 1D). In contrast, JNK1/2-deleted NEMO mice displayed ducto/cystogenesis that was much more pronounced than at earlier stages of CLD (Fig. 1B; Supporting Fig. S2D). NEMOΔhepa/JNKΔhepa livers exhibited significantly elevated glutamate dehydrogenase, total bilirubin, alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels compared with NEMOΔhepa mice, already detectable at 13 weeks (Fig. 1D,E; Supporting Fig. S2D,E). Altogether, these data indicated that deletion of Jnk1/2 in an experimental model of CLD has pivotal implications in cell death, cholestasis development, and ductular proliferation of cholangiocytes.

ANALYSIS OF THE MICROENVIRONMENT DRIVING MASSIVE BILE DUCT PROLIFERATION IN NEMOΔhepa/JNKΔhepa ANIMALS

Lack of NF-κB activity in hepatocytes triggers spontaneous HCC. In turn, 52-week-old
Fig. 1. Deletion of Jnk1/2 in 52-week-old NEMOΔhepa livers triggers cyst formation. (A) Macroscopic view of livers from 52-week-old NEMOΔf/f (wild type), NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice. (B) Representative H&E staining of liver sections of NEMOΔf/f, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa livers at 52 weeks of age. Different magnifications were used (left). Scale bar 200 µm. Cyst frequency and number of visible microscopic cysts per 10× view field were calculated and graphed (right), magnification is 10× for upper and magnification is 20× for lower. (C) BW (left); LW (center); LW/BW ratio (right). (D) Tumor burden for each individual mouse was characterized by calculating total number of visible tumors >5 mm in diameter per mouse (left); serum levels of GLDH (center); bilirubin (right). (E) Serum levels of AP (left), AST (center), and ALT (right), in 52-week-old NEMOΔf/f, JNKΔhepa, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice. Data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Abbreviations: AP, alkaline phosphatase; GLDH, glutamate dehydrogenase; H&E, hematoxylin and eosin.
NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} mice displayed significantly increased bile duct proliferation. We next investigated liver damage associated with the phenotype of these animals. We first applied terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining, which detects several types of cell death, including necrosis, apoptosis, and necroptosis.\textsuperscript{(20)} NEMO\textsuperscript{Δhepa} livers displayed a high percentage of TUNEL-positive cells, while NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} livers showed significantly increased levels of cell death (Fig. 2A).

Apoptosis and necroptosis are two relevant forms of cell death in the pathogenesis of human and murine liver disease.\textsuperscript{(21,22)} To discriminate between these two types of cell death, we performed immunohistochemistry (IHC) analyses, which revealed significantly higher levels of the apoptosis marker cleaved caspase 3 (CC3) in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} livers, especially in cholangiocytes but also in immune infiltrates of these livers (Fig. 2B). We subsequently studied proteins involved in necroptosis, e.g., receptor-interacting serine/threonine-protein kinase 3 (RIPK3) by western blot and IHC, which was found overexpressed in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} livers, while no difference in RIPK1 protein expression was found (Fig. 2E; Supporting Fig. S3A).

Cell-cycle dysregulation is characteristic of biliary overgrowth, resulting in CCA. Increased Ki-67-positive and proliferating cell nuclear antigen (PCNA)-positive cells were observed in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} frozen and paraffin sections, respectively, the latter further confirmed by immunoblot analysis (Fig. 2C,E; Supporting Fig. S3B). Moreover, the number of transcripts for PCNA and cyclin D1 was up-regulated in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} livers (Supporting Fig. S3C). P21 has been shown to induce cell-cycle arrest and promote the DNA repair gene, thus acting as a tumor suppressor.\textsuperscript{(23,24)} Interestingly, we observed p21 overexpression in Jnk1/2-deficient NEMO\textsuperscript{Δhepa} mice (Fig. 3E). Moreover, oxidative stress in the liver microenvironment has a definitive role in CCA development. We thus measured lipid peroxidation and the antioxidant defense of these animals. Interestingly, strong 4-hydroxynonenal immunostaining and catalase depletion (Supporting Fig. S3D,E) in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} livers confirmed elevated reactive oxygen species (ROS) production associated with cholangiocellular proliferation.

Hepatic stellate cells (HSCs) are important cells shaping the hepatic microenvironment after liver damage. Hence, HSCs are involved in the high number of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA)-positive myofibroblasts and extracellular matrix (ECM) deposition associated with CCA development.\textsuperscript{(25)} Sirius red staining and quantification confirmed strong collagen deposition in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} livers (Fig. 2D; Supporting Fig. S4A). Furthermore, collagen type I alpha 1 (Col1A1) and \(\alpha\)-SMA were dramatically overexpressed in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} liver protein lysates (Fig. 2E) and were associated with significantly increased transcripts of \(\text{col1a1}\), matrix metalloproteinase (\(\text{mmp}\))\textsubscript{7/9/12}, and tissue inhibitor of metalloproteinase (\(\text{timp}\))\textsubscript{1} (Supporting Fig. S4B,C). Cytokine-mediated tissue fibrosis was also measured, and levels of monocyte chemoattractant protein 1 (\(\text{mcp1}\)), tumor necrosis factor (\(\text{tnf}\)), interleukin (\(\text{il}\))\textsubscript{1\beta}, and regulated upon activation, normal T cell expressed, and secreted (\(\text{rantes}\)) were significantly up-regulated in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} mice (Supporting Fig. S4D,E).

Altogether, these data suggest that fibrogenesis and unresolved inflammation are involved in CCA development in the NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} context while HCC-related tumorigenesis is diminished as confirmed by low glutamine synthase expression (Supporting Fig. S5A).

### Table 1. Histopathological Characteristics of the Different Mouse Groups (Scale, 0-4)

| Characteristic          | NEMO\textsuperscript{Δhepa} | NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} |
|-------------------------|-------------------------------|----------------------------------------------------------|
| Neoplasia               | HCC                           | Cystic cholangioma                                        |
| Anisokaryosis           | 3.5                           | 2.0                                                      |
| Altered foci            | 2.0                           | 1.8                                                      |
| Mitosis/HPF (40×)       | 2.5                           | 1.0                                                      |
| Cellular hypertrophy    | 3.0                           | 2.5                                                      |
| Dysplasia               | 3.0                           | 2.5                                                      |
| Oval cell proliferation | 1.5                           | 2.5                                                      |
| Portal inflammation     | 1.0                           | 1.0                                                      |
| Overall inflammation    | 3.0                           | 3.5                                                      |
| Ductular reaction       | 1.5                           | 2.0                                                      |
| Apoptosis               | 1.0                           | 3.0                                                      |
| Fibrosis                | 3.0                           | 4.0                                                      |
| Steatosis               | 1.4                           | 0.0                                                      |
| Others                  | Pale cytoplasm hepatocytes    | Massive bile duct proliferation                          |

NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} Livers Exhibit Typical Features of Human CCA

Immunohistochemical analysis revealed that many ductules were composed of creatine kinase
Fig. 2. Characterization of cell death, cell proliferation, and collagen deposition in 52-week-old NEMOΔhepa/JNKΔhepa mice. (A) Representative TUNEL staining of liver sections of NEMOf/f, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa livers at 52 weeks of age (left). TUNEL-positive cells were quantified and graphed (right). (B) Representative IHC staining for CC3 of the same livers (left). CC3-positive cells were quantified and graphed (right). (C) Immunofluorescent staining for Ki-67 of liver cryosections from the same livers (left). Ki-67-positive cells were quantified and graphed (right). (D) Representative sirius red staining of paraffin sections from the indicated genotypes (left). Quantification of the positive sirius red area fraction was performed with Image J and graphed (right). (A-D) Scale bars, 200 μm. Arrows (→) indicate positive cells. Data are presented as mean ± SEM; ****P < 0.0001. (E) Protein levels of α-SMA, Col1A1, PCNA, p21, RIPK1, and RIPK3 from whole-liver extracts of 52-week-old NEMOf/f, JNKΔhepa, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice were analyzed by western blot with the indicated antibodies. GAPDH was used as a loading control.
(CK)19-positive cells. In fact, NEMO\textsuperscript{Ahepa}/JNK\textsuperscript{Ahepa} cystic and CCA-like structures exhibited CK19-positive staining that was corroborated by dramatically elevated CK7/19 messenger RNA (mRNA) expression in these livers (Fig. 3A,D). NEMO\textsuperscript{ff} had regular small bile ducts, and NEMO\textsuperscript{Ahepa} exhibited mild to
moderate ductular proliferates similar to a ductular reaction in humans. Noticeably, livers of NEMOΔhepa/JNKΔhepa mice displayed cholangiocellular proliferates with a tubular architecture resembling the morphology of human well-differentiated CCA, highlighted by a strong-positive CK19 signal. In contrast, hepatocyte nuclear factor (HNF)-4α staining was characteristic of pericystic areas and hepatocytes of NEMOΔhepa livers, which were associated with densely packed cholangiocellular proliferates with a tubular growth pattern mimicking morphologic features of well-differentiated human CCA (lower panel), magnification is 20×. (B) Representative IF staining for HNF-4α of the same livers, magnification is 20×. (C) Representative IHC staining for Muc1, magnification is 20×. (D) mRNA expression analysis of Ck7 (left), Ck19 and Muc5ac (center), and Gabrp (right) was quantified by qRT-PCR in the same livers. (E) mRNA expression analysis of Epcam (left), Cdk133 and Muc1 (center), and Dmbt1 (right) and Gabrp receptor, pi (gabrp), and gamma-aminobutyric acid receptor, a (gabaRa), and immunoglobulin heavy chain (gamma chain) (gamgg)Δhepa/JNKΔhepa mice were analyzed by western blot with the indicated antibodies. GAPDH served as loading control. Data are presented as mean ± SEM. *P < 0.05; **P < 0.001. Abbreviations: CD, cluster of differentiation; Epcam, epithelial cell adhesion molecule; IF, immunofluorescence.

FIG. 3. Loss of Jnk1/2 in NEMOΔhepa hepatocytes triggers cholangiocellular proliferation. (A) Representative IF for CK19 of liver cryosections was performed in 52-week-old NEMOΔnf, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa livers (upper panel). IHC staining for CK19 reveals densely packed cholangiocellular proliferates with a tubular growth pattern mimicking morphologic features of well-differentiated human CCA (lower panel), magnification is 20×. (B) Representative IF staining for HNF-4α of the same livers, magnification is 20×. (C) Representative IHC staining for Muc1, magnification is 20×. (D) mRNA expression analysis of Ck7 (left), Ck19, and Muc5ac (right) was quantified by qRT-PCR in the same livers. (E) mRNA expression analysis of Epcam (left), Cdk133 and Dmbt1 (center), and Gabrp (right) was quantified by qRT-PCR of samples taken from NEMOΔnf, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa/JNKΔhepa tumors killed at 52 weeks. (F) Protein expressions of Gabrp and Dmbt1 from whole-liver extracts of 52-week-old NEMOΔnf, JNKΔhepa, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice were analyzed by western blot with the indicated antibodies. GAPDH served as loading control. Data are presented as mean ± SEM. *P < 0.05; **P < 0.001. Abbreviations: CD, cluster of differentiation; Epcam, epithelial cell adhesion molecule; IF, immunofluorescence.

To validate the relevance of Jnk1/2 in mediating the shift from HCC to CCA, we applied a second model of carcinogenesis, the DEN model. Previously, Das and colleagues, using mice with compound deficiency of Jnk1/2 in hepatocytes, demonstrated that the JNK genes possess tumor-suppressing roles in liver carcinogenesis that depend on the cell types of the liver. Additionally, we showed that hepatocyte-specific Jnk1/2 knockout female and male mice have no phenotype affecting the correct function of the liver. These previous results are confirmed in the present study in a larger pool of animals ranging from 13 to 52 weeks of age (Supporting Figs. S6A-D and S7A-D). However, approximately 33% of these mice from week 30 of age histologically displayed tumors resembling human CCA in their liver parenchyma that did not affect liver function (Fig. 1A,B; Supporting Fig. S7A-D).

JNKΔhepa mice were challenged with the carcinogen DEN. Interestingly, these mice exhibited jaundice and the liver was yellowish albeit with decreased tumor load (Supporting Fig. S8A). The LW/BW ratio was significantly decreased in JNKΔhepa compared with JNKΔnf mice (Supporting Fig. S8B). Histologic evaluation performed by two blinded pathologists demonstrated the presence of cystogenesis and cholangioma-like structures in liver parenchyma accompanied by strong infiltration of immune cells (Fig. S8C). The analysis of serum transaminases in JNKΔhepa demonstrated decreased ALT and AST levels in these animals (Fig. S8D,E). These results indicated that JNK1 and JNK2 might influence cell fate during liver tumorigenesis.

To further analyze the differences between both animal models, age progression versus chemically induced HCC, we performed a microarray analysis (Supporting Fig. S9A,B). Interestingly, dmbt1, muc1, gabrp, and immunoglobulin heavy chain (gamma polypeptide) (igvh)2b were commonly up-regulated in Jnk1/2-deficient NEMOΔhepa mice and Jnk1/2-deficient mice challenged with DEN, indicating that epithelial–mesenchymal transition (EMT) might be modulated through a JNK-dependent mechanism.

ADMINISTRATION OF DEN TO JNKΔhepa MICE TRIGGERS HEPATIC DUCTO/CYSTOGENESIS WITHOUT HEPATOCARCINOCGENESIS

To validate the relevance of Jnk1/2 in mediating the shift from HCC to CCA, we applied a second model of carcinogenesis, the DEN model. Previously, Das and colleagues, using mice with compound
Next, we analyzed total common up-regulated and down-regulated genes in NEMOΔhepa/JNKΔhepa versus JNKΔhepa + DEN, which were the majority compared to specific changes of each experimental model (Supporting Fig. S9C,D). Heat map analysis of these genes showed up-regulation of genes related to CCA, like dmbt1, muc1, gabrp, and matrix deposition, including timp1 and mmp12, and down-regulation of serpines, which are often implicated in HCC development (Fig. 4A). Altogether, these data reinforce the notion that the JNK signaling pathway modulates cell fate during liver carcinogenesis.

OVEREXPRESSION OF THE IL-6/SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 PATHWAY IS CHARACTERISTIC FOR NEMOΔhepa/JNKΔhepa LIVERS

Cytokines play an important role during carcinogenesis by shaping the inflammatory microenvironment toward malignant transformation. In particular, IL-6 has been demonstrated to have an integral role in CCA biology and other cancers as a growth and survival factor. (28) Under physiologic conditions, IL-6 through a Janus kinase (JAK)/signal transducer and activator of transcription (STAT) 3 pathway induces expression of suppressor of cytokine signaling 3 (SOCS3), accelerating inflammation, cell growth, and tumor formation. We found overexpression of il6, il6 receptor (il6r), and socs3 mRNA as well as other IL-6 family members, including LIF IL-6 family cytokine (lif) and oncostatin M (osm) (Fig. 4B,C; Supporting Fig. S10A). OSM has recently been identified as an important regulator of the EMT/cancer stem cell plasticity program that promotes tumorigenic properties. (29) Osm was significantly increased in NEMOΔhepa/JNKΔhepa livers.

Concomitantly, high phosphorylated STAT3 (pSTAT3) levels were evident in NEMOΔhepa/JNKΔhepa compared with NEMOΔhepa livers (Fig. 4D; Supporting Fig. S10B), indicating that this pathway is strongly activated in our murine model.

THE EGF-Raf-MEK-ERK1/2 PATHWAY IS OVEREXPRESSED IN NEMOΔhepa/JNKΔhepa ANIMALS

Activation of Notch and Wnt signaling pathways stimulates proliferation of the hepatic progenitor cell compartment. In particular, NOTCH signaling is implicated in the commitment toward the cholangiocyte fate, while WNT can trigger differentiation toward the hepatocyte lineage. (30) Therefore, we first assessed the relevance of Notch-1 (A6) in NEMOΔhepa/JNKΔhepa livers. Interestingly, A6 staining was positive in clusters of immune cells in NEMOΔhepa livers. NEMOΔhepa/JNKΔhepa livers exhibited increased Notch-1 expression throughout the liver parenchyma (Fig. 5A). In fact, other family members, including Notch2, were significantly up-regulated or had a tendency toward increased mRNA levels in NEMOΔhepa/JNKΔhepa livers (Fig. 5C; Supporting Fig. S11A-F). However, no differences in β-catenin expression between NEMOΔhepa and NEMOΔhepa/JNKΔhepa livers were observed (Fig. 5D).

Of note, JNK1/2-knockout mice had reduced phosphorylation of β-catenin, indicating that JNK is necessary for β-catenin phosphorylation, as suggested. (31)

The epidermal growth factor receptor (EGFR) family includes erythroblastic oncogene B (ERBB1), 2, 3, and 4, with ERBB1/EGFR and ERBB2/human epidermal growth factor receptor 2 (HER2) (Neu in rodents) being frequently implicated in the multistep carcinogenesis of CCA. (32) Specifically, HER2 is a well-described predictive biomarker for positive anti-HER2 therapy response in breast and gastric cancer and, lately, in CCA. (33,34) Our first results showed dramatically increased ErbB2 protein and mRNA levels in livers of NEMOΔhepa/JNKΔhepa compared with NEMOΔhepa hepatic tissue (Fig. 5B-D). Interestingly, we also found increased expression of the EGFR ligand egf (Fig. 5C). Consistently, the levels of EGFR phosphorylation (pEGFR) were markedly elevated in the livers of NEMOΔhepa/JNKΔhepa mice (Fig. 5D).

The rapidly accelerated fibrosarcoma (RAF)-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) transduction pathway is a key signaling cascade that regulates cellular proliferation, differentiation, and apoptosis and is frequently dysregulated in HCC (35) and in biliary tract cancer. (36) Binding of EGF to EGFR triggers its tyrosine kinase activity downstream signaling leading to the end phosphorylation of MEK1/2 and ERK1/2, with translocation of ERK to the nucleus and expression of genes related to proliferation. Increased phosphorylation (i.e., activation) of RAF, MEK1/2, and ERK1/2 was
found in NEMO\textsuperscript{Δhepa}/JNK\textsuperscript{Δhepa} mice (Fig. 5D) associated with biliary overgrowth. These results were further corroborated in livers of 13-week-old mice (Supporting Fig. S12), indicating that the RAF-MEK-ERK pathway was constitutively activated at early stages of cholangiocarcinogenesis.
FIG. 4. The IL-6/STAT3 pathway is pivotal in biliary cell proliferation during liver carcinogenesis. (A) Gene array analysis was performed in 8-week-old NEMOΔhepa/JNKΔhepa and NEMOΔhepa/JNKΔhepa mice in addition to 26-week-old wild-type and JNKΔhepa and JNKΔhepa livers challenged with DEN. Correlation of the fold induction of gene expression in liver is shown. Log2 expression values of the individual mice were divided by the mean of the NEMOΔhepa/JNKΔhepa mice. Log ratios were saved in a .txt file and analyzed with the Multiple Experiment Viewer. Top up-regulated and down-regulated target substrates are shown (red, up-regulated; green, down-regulated; n = 3, 3.0< fold change >3.0). (B) mRNA expression analysis of il6 (left); il6r (center), and socs3 (right). (C) mRNA expression analysis of osm (left), osmr (center), and pJnk (right) was quantified by qRT-PCR of samples taken from NEMOΔhepa, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa livers killed at 52 weeks. (D) Protein expression levels of STAT3 and pSTAT3 from whole-liver extracts of 52-week-old NEMOΔhepa, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice were analyzed by western blot with the indicated antibodies. GAPDH served as loading control. Abbreviations: DKO, JNK1f/f/JNK2−/− + DEN; LIF, leukemia inhibitory factor; osmr, oncostatin M receptor; TKO, NEMOΔhepa/JNKΔhepa.

LAPATINIB TREATMENT PREVENTS BILIARY EXPANSION AND DUCTO/CYSTOGENESIS IN NEMOΔhepa/JNKΔhepa MICE

Our data strongly indicate the EGFR-HER2 pathway being involved in biliary cell hypergrowth and cholangiocarcinogenesis in NEMOΔhepa/JNKΔhepa livers. Lapatinib is a dual EGFR2/HER2 tyrosine kinase inhibitor (TKI) targeting both EGFR and HER2. It successfully inhibited the growth of HER-overexpressing breast cancer cells in culture and in tumor xenografts. Taking into consideration the promising therapeutic option of lapatinib, we subsequently explored the potential beneficial effect of this dual TKI in our model of cholangiocellular proliferation.

Lapatinib was administered daily by oral gavage for 6 weeks in NEMOΔhepa, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice (Fig. 6A). The TKI did not cause any relevant histopathological or serum biochemistry alterations to NEMOΔhepa or NEMOΔhepa animals (Fig. 6B). However, lapatinib treatment of NEMOΔhepa/JNKΔhepa mice resulted in a significant reduction of cyst-like structures (Fig. 6B). Lapatinib also significantly reduced serum AST and ALT levels in NEMOΔhepa/JNKΔhepa compared with NEMOΔhepa and NEMOΔhepa animals (Fig. 6C).

Next, the impact of lapatinib on EGFR/HER2 signaling was tested by investigating the downstream RAF-MEK-ERK pathway below EGFR/HER2. Decreased activation of EGFR and abrogation of RAF, MEK, and ERK signaling pathways were found in lapatinib-treated livers compared with vehicle-administered NEMOΔhepa/JNKΔhepa livers (Fig. 6D). These results suggest that lapatinib successfully decreases EGFR-HER2 signaling and functionally links inhibition of this pathway with biliary overgrowth in NEMOΔhepa/JNKΔhepa livers.

COMBINED JNK1/JNK2 DELETION IS ESSENTIAL FOR HYPERPROLIFERATION OF BILE DUCTS

Our data were generated in global Jnk2−/− mice. Hence, we aimed to distinguish if hepatocytic or nonparenchymal JNK2 function is essential to direct bile duct proliferation. We applied our recently developed hepatocyte-specific Jnk2 siRNA (siJnk2) protocol using lipid nanoparticles; siLuc served as controls. We included 6- to 8-week-old untreated floxed NEMOΔhepa/JNK1Δhepa, siLuc-treated NEMOΔhepa/JNK1Δhepa, and siJnk2-challenged NEMOΔhepa/JNK1Δhepa mice in this analysis. In parallel, animals were treated with vehicle or lapatinib.

Interestingly, Jnk2 knockdown in hepatocytes triggered massive biliary cyst formation in livers of vehicle-treated NEMOΔhepa/JNK1Δhepa animals compared with siLuc-NEMOΔhepa/JNK1Δhepa and untreated floxed NEMOΔhepa/JNK1Δhepa mice (Supporting Fig. S13A). These results demonstrate that combined Jnk1/2 deletion in hepatocytes is responsible for directing biliary hyperproliferation in this model. Additionally, lapatinib treatment successfully reduced liver cystogenesis and significantly ameliorated serum transaminases in NEMOΔhepa/JNK1Δhepa + siJnk2 livers, confirming the efficacy of this TKI in experimental biliary carcinogenesis (Supporting Fig. S13B-D).

Discussion

Hyperplasia of the biliary epithelia with variable atypia in cystic bile ducts may give rise to malignant transformation leading to intrahepatic CCA for which an enormous unmet clinical and research need exists. CCA is an epithelial neoplasm derived from
Fig. 5. Activation of EGF-EGFR-RAF-MEK1/2-ERK1/2 is distinctive of NEMOΔhepa/JNKΔhepa mice. (A) Representative IHC for NOTCH-1 staining of liver sections of 52-week-old NEMOff, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa livers, magnification is 20×. (B) Representative IHC for ErbB2 staining of liver sections of the same livers. (C) mRNA expression analysis of notch-2 (left), erbB2 (center), and egf (right) was quantified by qRT-PCR of samples taken from NEMOff, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice killed at 52 weeks. Data are presented as mean ± SEM. *P < 0.05; ****P < 0.0001. (D) Protein expressions of phospho-β-catenin, β-catenin, ErbB2, phospho-EGFR, phospho-RAF, phospho-MEK1/2, phospho-ERK1/2, and total ERK1/2 from whole-liver extracts of 52-week-old NEMOff, JNK1Δhepa/JNK2−/−, NEMOΔhepa, and NEMOΔhepa/JNKΔhepa mice were analyzed by western blot with the indicated antibodies. GAPDH served as loading control. Abbreviation: phospho, phosphorylated.
primary and secondary bile tracts and accounts for 5%-10% of primary liver cancer, the incidence and mortality of which are steadily increasing. The 5-year survival of patients with CCA remains unacceptably low, and survival has not dramatically improved in the past 20 years. This is in part due to a lack of...
understanding of the pathophysiologic mechanisms underlying CCA. Because biliary tract cancer is often diagnosed late, the success of the only curative procedure (surgical resection) is limited, and the lack of biomarkers or diagnostic tools that would lead to early diagnosis is a matter of concern.(41)

A recent study(12) opened a Pandora’s box for therapeutic options targeting the JNK signaling pathway, a major regulator of cell proliferation, against CCA. Earlier, several studies confirmed the critical role of the JNK signaling pathway in liver cancer.(10,19,42-44) At present, little is known on the role of JNK in directing differentiation of LPCs and more specifically of bipotential hepatic cells not only in liver homeostasis but also following liver injury.

We first focused on the specific roles of the Jnk genes in the progression of experimental chronic liver disease using NEMO mice and showed how Jnk1 or Jnk2 tipped the balance toward HCC or necro-inflammation, respectively.(19) We later demonstrated that combined Jnk1/2 deletion in hepatocytes triggers more severe liver injury, inflammation, and progression after toxic liver injury.(15)

Thus, we next sought to investigate the consequences of hepatocytic Jnk1/2 ablation in liver parenchymal proliferation and growth in experimental HCC. For this purpose, we generated NEMO/JNK mice, which displayed reduced tumor burden, despite the fact that they had signs of jaundice and hyperbilirubinemia, compared to NEMO-deficient mice developing HCC.(18) Remarkably, Jnk1/2-deleted NEMO/JNK livers exhibited hyperproliferation of the biliary epithelium, forming cyst-like structures compatible with cholangioma or malignant CCA, as assessed by two independent pathologists (Table 1).

These livers were characterized by cell death, inflammatory microenvironment, and ECM deposition. Necroptosis-associated hepatic cytokine microenvironment induces the shift from HCC to CCA development.(22) NEMO/JNK mice display considerable CC3 staining and RIPK3 protein levels, triggering exacerbated compensatory proliferation of LPCs, which are associated with increased ROS production and failure of the antioxidant defense.

Moreover, deposition of ECM and periductal/pericystic scar formation was a prominent feature in NEMO/JNK livers. A unique characteristic of CCA is the presence of cancer-associated fibroblasts (CAFs) surrounded by numerous immune cells.(41) CAFs promote the secretion of chemokines/cytokines including EGF in CCA cell lines.(45) Moreover, TNF might be another important culprit in CCA development, as suggested.(12)

In parallel with proliferating hepatocytes and a marked expansion of the biliary epithelium, the liver parenchyma of NEMO/JNK animals was positive for CK19 and SOX-9 and negative for HNF-4α. Moreover, we found increased expression of mucin genes. Considering that CCA tissues are characterized by the presence of mucin-secreting cells, this finding further supports the CCA diagnosis.(46)

Interestingly, analysis of gene expression showed the occurrence of CCA/epithelial-transformed neoplasia-enriched markers, including Dmbt1 and Gabrp, not only in NEMO/JNK liver but also in a second model of liver carcinogenesis, the DEN model. Confirmation in the two models suggests that CCA development relies on Jnk1/2 combined function in hepatocytes but is independent of NF-κB activity in LPCs. Moreover, microarray studies highlighted the pivotal role of Jnk1/2 in modulating cell fate by promoting hepatocarcinogenesis and under-regulating cascades linked with cholangiocarcinogenesis. Our data undoubtedly indicate that loss of Jnk1/2 function promoted CCA in both experimental CLD and chemically induced HCC.

Jnk1/2-deleted NEMO livers exhibited strong expression of NOTCH-1/A6 and Notch-2 and a clear tendency toward increased expression of Notch signaling pathway effectors. These results are consistent
with evidence from mouse studies, suggesting that NOTCH and WNT/β-catenin are key drivers of CCA development. Specifically, NOTCH-1, -2, and -3 were shown to be overexpressed in human cholangiocellular injury. In contrast, no differences in β-catenin expression between NEMOΔhepa and NEMOΔhepa/JNKΔhepa were observed, indicating that hepatocyte differentiation in our model is inhibited in favor of ductular reaction and cholangiocyte differentiation. Of note, JNK1/2-knockout mice have reduced phosphorylation of β-catenin, suggesting that JNK is necessary for β-catenin phosphorylation, as suggested. Our protein and mRNA data overwhelmingly indicated that overexpression and activation of the EGFR/ErbB2 family was implicated in multistep cystogenesis toward CCA in NEMOΔhepa/JNKΔhepa livers. Indeed, EGFR overexpression occurs in 11%-27% of human CCA, whereas HER2 overexpression is less frequent but very characteristic in transgenic mouse models. It has been reported that EGF is released by tumor-associated macrophages (TAM). Binding of EGF to its receptors induces their homodimerization or heterodimerization, which in turn activates downstream signaling pathways that regulate cell differentiation, migration, angiogenesis, and survival.

In our study, we specifically focused on RAF-MEK1/2-ERK2 and JAK/STAT signaling. Both pathways were dramatically induced in NEMOΔhepa/JNKΔhepa livers, suggestive of the strong proliferative and inflammatory microenvironment within the hepatic parenchyma. Therefore, we tested the possibility of blocking EGFR/HER2 signaling as a novel strategy in treating hyperproliferation of the biliary epithelium. We used lapatinib, a dual TKI that efficiently inhibits both EGFR and HER2. Treatment not only prevented RAF-MEK1/2-ERK2 activation but also inhibited biliary cell hyperproliferation. It also defines new therapeutic options to inhibit pathways involved in cholangiocarcinogenesis.

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Author names in bold designate shared co-first authorship.

Supporting Information

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