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c-Met Confers Protection Against Chronic Liver Tissue Damage and Fibrosis Progression After Bile Duct Ligation in Mice

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BACKGROUND & AIMS: The hepatocyte growth factor (HGF)/mesenchymal-epithelial transition factor (c-Met) system is an essential inducer of hepatocyte growth and proliferation. Although a fundamental role for the HGF receptor c-Met has been shown in acute liver regeneration, its cell-specific role in hepatocytes during chronic liver injury and fibrosis progression has not been determined. **METHODS:** Hepatocyte-specific c-Met knockout mice (c-Met$^{\Delta hepa}$) using the Cre-loxP system were studied in a bile duct ligation (BDL) model. Microarray analyses were performed to define HGF/c-Met–dependent gene expression. **RESULTS:** Two strategies for c-Met deletion in hepatocytes to generate hepatocyte-specific c-Met knockout mice were tested. Early deletion during embryonic development was lethal, whereas post-natal Cre expression was successful, leading to the generation of viable c-Met$^{\Delta hepa}$ mice. BDL in these mice resulted in extensive necrosis and lower proliferation rates of hepatocytes. Gene array analysis of c-Met$^{\Delta hepa}$ mice revealed a significant reduction of anti-apoptotic genes in c-Met–deleted hepatocytes. These findings could be tested functionally because c-Met$^{\Delta hepa}$ mice showed a stronger apoptotic response after BDL and Jo-2 stimulation. The phenotype was associated with increased expression of proinflammatory cytokines (tumor necrosis factor-α and interleukin-6) and an enhanced recruitment of neutrophils. Activation of these mechanisms triggered a stronger profibrogenic response as evidenced by increased transforming growth factor-β_1, α-smooth muscle actin, collagen-1α messenger RNA expression, and enhanced collagen-fiber staining in c-Met$^{\Delta hepa}$ mice. **CONCLUSIONS: Our results show that deletion of c-Met in hepatocytes leads to more liver cell damage and fibrosis in a chronic cholestatic liver injury model because c-Met triggers survival signals important for hepatocyte recovery.**

Chronic liver damage can be triggered by different mechanisms (eg, viral hepatitis, metabolic liver diseases, or chronic alcohol consumption).[1] Because of chronic insults the liver activates a uniform etiology-independent program, which results in collagen deposition and, finally, liver fibrosis.[2] In human beings liver fibrosis is associated with high morbidity and mortality. At present, no therapeutic concepts have been developed to treat and reverse fibrosis.

Animal models of liver fibrosis are essential to study and understand the molecular mechanisms of hepatic fibrosis, which might be relevant to establish new treatment options.[1] In the bile duct ligation (BDL) model, biliary obstruction leads to damage of the biliary epithelium and later also hepatocytes. In addition, inflammatory and tissue damage[3]–mediating growth factors, cytokines, and chemokines are induced. Moreover, quiescent hepatic stellate cells are activated and transformed into matrix-producing myofibroblasts.[2] These cells produce high amounts of extracellular matrix, but also secrete enzymes that inhibit collagen degradation, which eventually results in liver fibrosis.

Hepatocyte growth factor (HGF) has been discovered and cloned[4] because of its strong mitogenic potential for hepatocytes during liver regeneration.[5] HGF binds its membrane receptor mesenchymal-epithelial transition factor (c-Met), triggering its dimerization and phosphorylation. Thus, intracellular adapter proteins activating distinct intracellular signals, eg, the PI3K, Ras, and ERK pathways,[6,7] are bound to c-Met and execute promitogenic and anti-apoptotic functions described for HGF.[8] In earlier studies an antifibrotic effect has been reported in rodent fibrosis models. Thereby, HGF was adminis-

Abbreviations used in this paper: AFP, α-fetoprotein; Alb-Cre, albumin promoter–Cre transgenic animals; Alfp-Cre, albumin/AFP promoter/enhancer; BDL, bile duct ligation; BrdU, bromodeoxyuridine; c-Met, mesenchymal-epithelial transition factor; DAPI, 6-diamidino-2-phenylindole; HGF, hepatocyte growth factor; HSC, hepatic stellate cell; IKK, inhibitor of nuclear factor kappa; MMP-13, matrix metalloproteinase 13; PDGF-D, platelet derived growth factor D; STAT3, signal transducer and activator of transcription 3; TIMP-1, tissue inhibitor of matrix metalloproteinase 1; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFAIP2, tumor necrosis factor alpha induced protein 2; TNFAIP3, tumor necrosis factor alpha induced protein 3; TRAIL, TNF-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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tered continuously and resulted in a significant reduction of excessive production and deposition of extracellular matrix.[9]

c-Met knockout animals die during embryonal development in utero.[4,10] Therefore, we previously used the ubiquitously active, interferon-dependent Mx1-promoter, which allowed the deletion of c-Met in adult mice.[11] Partial hepatectomy in these animals resulted in impaired liver regeneration and could be characterized as a defect in Erk1/2 kinase activation.

In the present study we now aimed to address and define more specifically the role of the HGF/c-Met system in hepatocytes. Therefore, we generated hepatocyte-specific c-Met knockout mice and used these animals in a liver fibrosis model to elucidate the potential cytoprotective effects of HGF. Those data have been strengthened by microarray analysis from HGF-treated mice to better define c-Met–dependent signaling in vivo.

Materials and Methods

Generation of Hepatocyte-Specific c-Met Knockout Mice

c-Met$^{loxP/loxP}$ animals[11] were first crossed with albumin/α-fetoprotein (AFP) promoter/enhancer[12] (Alfp-Cre) or albumin promoter–Cre transgenic animals[13] (Alb-Cre). The Alfp-Cre animals express Cre-recombinase during embryonal development, whereas Alb-Cre mice start to express Cre 2 weeks after birth.

BDL

Eight- to 10-week-old mice were subjected to BDL as indicated. BDL was performed by tying the common bile duct using a nonabsorbable filament (Ethicon, Boston, MA). For each of the following analyses at least 4 mice per genotype and time point were investigated. All experiments were performed at least twice.

Histology and Immunoblotting Analysis

A detailed description of the histochemical, immunohistologic, and immunoblotting techniques and methods used is provided in Supplementary Materials and Methods.

Gene Expression Analysis by Real-Time Polymerase Chain Reaction and Microarrays

Total RNA was extracted from cryopreserved liver tissue by using peqGold RNAPure (PeqLab, Erlangen, Germany). After isolation, 500 ng of total RNA was transcribed into cDNA by using the RT Omniscript Kit (Qiagen, Hilden, Germany). The detection of cDNA expression for the specific genes was performed by using the SybrGreenER quantitative polymerase chain reaction Supermix (Invitrogen, Karlsruhe, Germany).

Primer sequences and a detailed methods section for the microarray analysis are provided in Supplementary Materials and Methods and Supplementary Table 4.

Evaluation of Statistical Significance

All significant *P* values were calculated and proven via the Student *t* test.

Results

Embryonal Hepatocyte-Specific c-Met Deletion Is Lethal

We first aimed to generate hepatocyte-specific c-Met conditional knockout animals. c-Met$^{loxP/loxP}$ animals were crossed with transgenic animals expressing the Cre-recombinase under the control of an Alfp-Cre construct. Our earlier results showed that this construct results in efficient target gene deletion in hepatocytes at day 12 during embryonic development.[14] Interestingly, cross-breeding of c-Met$^{loxP/loxP}$ animals with Alfp-Cre(+/−)/c-Met$^{loxP/wt}$ mice did not result in hepatocyte-specific c-Met knockout animals because no viable Alfp-Cre(+)/c-Met$^{loxP/loxP}$ mice were born (Figure 1*A*). This supports prior findings[5] that the HGF/c-Met system in hepatocytes is essential for normal mouse embryonic development.

As an alternative strategy we used an Alb-Cre construct expressing Cre-recombinase in hepatocytes only after birth. This strategy was successful in generating a hepatocyte-specific c-Met knockout mouse line (c-Met$^{\Delta hepa}$). Offspring of c-Met$^{\Delta hepa}$ and c-Met$^{loxP/loxP}$ animals were born in a normal Mendelian frequency (Figure 1*A*) and showed a deletion efficiency of c-Met in hepatocytes of more than 95% (Figure 1*B*). The functional effect of this deletion was evidenced further by showing a lack of c-Met phosphorylation in primary hepatocytes derived from c-Met$^{\Delta hepa}$ mice (Figure 1*C*).

Hepatocyte-Specific Deletion of c-Met Results in Enhanced Necrosis and Fibrosis After BDL

We now performed BDL in c-Met$^{\Delta hepa}$ and c-Met$^{loxP/loxP}$ (controls) mice to test the relevance of c-Met signaling in a model of chronic liver injury. All control mice survived the first 4 weeks after BDL, whereas 15% of c-Met$^{\Delta hepa}$ animals died during this time period (Figure 2*A*). Serum transaminase levels increased after BDL in both groups (Figure 2*B*), although 4 weeks after BDL a strong and significant decrease in serum protein levels was found in c-Met$^{\Delta hepa}$ mice compared with the control group (Figure 2*C*).

To better characterize the impact of c-Met deletion on liver injury we next performed H&E stainings of liver sections. In c-Met$^{\Delta hepa}$ mice significant more necrosis (higher numbers and larger areas) was found 3 and 7 days after BDL compared with c-Met$^{loxP/loxP}$ controls (Figure 2*D* and *E*). A quantification of necrotic areas showed on average 4–10 times more (and larger) necrotic areas in c-Met$^{\Delta hepa}$ livers. In addition, more infiltrating mononuclear cells were evident in bile duct–ligated c-Met$^{\Delta hepa}$ mice.

Twenty-eight days after BDL more dramatic histomorphologic changes were evident in the c-Met$^{\Delta hepa}$ group.