Hepatic stellate cell-expressed endosialin balances fibrogenesis and hepatocyte proliferation during liver damage

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Abstract

Liver fibrosis is a reversible wound-healing response to injury reflecting the critical balance between liver repair and scar formation. Chronic damage leads to progressive substitution of liver parenchyma by scar tissue and ultimately results in liver cirrhosis. Stromal cells (hepatic stellate cells [HSC] and endothelial cells) have been proposed to control the balance between liver fibrosis and regeneration. Here, we show that endosialin, a C-type lectin, expressed in the liver exclusively by HSC and portal fibroblasts, is upregulated in liver fibrosis in mouse and man. Chronic chemically induced liver damage resulted in reduced fibrosis and enhanced hepatocyte proliferation in endosialin-deficient (ENKO) mice. Correspondingly, acute-liver-damage-induced hepatocyte proliferation (partial hepatectomy) was increased in ENKO mice. A candidate-based screen of known regulators of hepatocyte proliferation identified insulin-like growth factor 2 (IGF2) as selectively endosialin-dependent hepatocyte mitogen. Collectively, the study establishes a critical role of HSC in the reciprocal regulation of fibrogenesis vs. hepatocyte proliferation and identifies endosialin as a therapeutic target in non-neoplastic settings.

Keywords angiocrine signaling; endosialin; liver fibrosis; liver regeneration

Subject Categories Digestive System; Immunology; Vascular Biology & Angiogenesis

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Introduction

Nutritive–toxic, metabolic, or infectious challenges result in acute or chronic liver damage, affecting millions of patients worldwide (Poynard et al., 2010). Chronic liver damage leads to liver fibrosis (Lee & Friedman, 2011) that may progress to cirrhosis (Rahimi & Rockey, 2012), which is a major risk factor for hepatocellular carcinoma (HCC) (Rahimi & Rockey, 2011). A hallmark of liver fibrosis is the proliferation and transdifferentiation of hepatic stellate cells (HSC), which also serve as major producer of fibrogenic extracellular matrix (Hernandez-Gea & Friedman, 2011). Targeting HSC activation is therefore considered a promising approach to reduce liver fibrosis and the risk of cirrhosis (Ebrahimkhani et al., 2011; Inagaki et al., 2012). HSC also affects hepatocyte proliferation through paracrine signaling mechanisms and thereby contributes to liver regeneration (Ebrahimkhani et al., 2011). Similarly, endothelial cells control liver regeneration (Ding et al., 2010; Hu et al., 2014) and the concepts of endothelial angiocrine signaling have recently been expanded to liver fibrosis (Ding et al, 2014). Thus, liver fibrosis and hepatocyte proliferation appear to be reciprocally regulated processes.

The C-type lectin-like transmembrane protein endosialin was originally identified as marker of angiogenic endothelial cells (Rettig et al., 1992; St Croix et al., 2000; Christian et al., 2001). However, later work by us and others revealed that endosialin is not a marker of angiogenic endothelial cells, but rather a marker of activated pericytes and tumor stromal myofibroblasts (Rettig et al., 1992; St Croix et al., 2000; MacFadyen et al., 2005, 2007; Christian et al., 2008; Simonavicius et al., 2008). Based on its strict oncofetal expression, endosialin has consequently been proposed as a tumor stroma therapeutic target. In fact, clinical trials with an endosialin antibody (MORAb-004) are ongoing (www.clinicaltrials.gov/ct2/results)
term = endosialin&Search = Search). Likewise, endosialin has been proposed as a target for tumor immune-PET applications (Chacko et al., 2014) and for tumor vasculature targeting vaccination strategies (Faciponte et al., 2014).

Based on the exclusive expression of endosialin by activated pericytes and tumor stromal myofibroblasts and also stimulated by ongoing efforts to translate endosialin as a therapeutic target, we hypothesized that endosialin may be a marker of activated hepatic stellate cells, which are the organ-specifically specialized pericytes of the liver. This study was consequently aimed at analyzing the role of endosialin during liver fibrosis and liver regeneration.

Results and Discussion

Upregulation of endosialin expression by activated hepatic stellate cells in early liver fibrogenesis

Corresponding to the reported expression of EN by activated mesenchymal cells including pericytes and (myo)fibroblasts (Christian et al., 2008), weak expression restricted to HSC and portal fibroblasts was detected in healthy adult murine and human livers (Fig 1A–C and E). EN was strongly upregulated in human liver fibrosis (FL) and active cirrhosis (CL+) showing immunohistochernical overlap with well-established pericyte and myofibroblast markers (such as PDGFR beta or α-smooth muscle actin) but never with endothelial markers (Fig 1D, F and G; Supplementary Figs S1 and S2). Downregulated EN expression during late-stage cirrhosis (Fig 1D and H) identified EN as an early HSC activation marker. Comparative analysis of EN expression with the established HSC activation marker α-smooth muscle actin (αSMA) identified a substantially stronger differential of EN expression in diseased vs. healthy tissue (Fig 1I and M versus J and N). Correspondingly, qRT–PCR analyses determined a significant change in the ratio of EN-to-αSMA mRNA expression comparing normal with fibrotic or cirrhotic liver (Fig 1O). EN expression was also more pronounced in early stages of fibrogenesis compared to collagen 1α, the main product of HSC in fibrotic tissue (Fig 1P).

Reduced fibrosis in EN-deficient mice in chronic liver damage

To study the consequences of acute liver damage in vivo, hepatotoxicity was induced in mice by injection of a single dose of carbon tetrachloride (CCL₄). Acute liver toxicity led to rapid upregulation of EN and αSMA (Fig 2A and B), followed by upregulated collagen 1α expression (Supplementary Fig S3). Analysis of isolated cell populations identified the vitamin A⁺, early activated HSC population (8.26% density gradient layer) as the primary EN-positive cell population (Fig 2C).

Based on the observed prominent regulation of EN in a mouse model of acute liver damage, we next performed comparative hepatotoxicity experiments in wild-type (WT) and EN-deficient mice (ENKO). ENKO mice are viable and fertile and display no overt phenotype unless they are pathologically challenged (Nanda et al., 2006). Likewise, comparative analyses of WT and ENKO livers from adult mice showed no overt morphological and liver-related clinical chemistry alterations (Supplementary Fig S4). A single high-dose injection of CCL₄ led to an equal extent of liver damage with acute zonal necrosis and increase in serum liver enzymes in both genotypes (Supplementary Fig S5). Twice weekly chronic CCL₄ administration resulted in a continuous increase in EN expression in WT mice over a period of 6 weeks (Fig 2D). Overall liver damage (assessed by cleaved caspase-3 activity) was similar in WT and ENKO mice (Supplementary Fig S6; Fig 2F and G). Likewise, the initiation of liver fibrosis was comparable in WT and ENKO mice as indicated by similar levels of αSMA and desmin expression (Supplementary Fig S7). Yet, fibrosis progression was delayed in ENKO mice as evidenced by significantly ameliorated (necro-)inflammatory activity (assessed by METAVIR Score), reduced levels of collagen 1α, PDGFR beta, αSMA, desmin, and TIMP1 expression as well as lower liver serum enzymes after 6 weeks of repeated CCL₄ administration (Fig 2E–M; Supplementary Figs S8, S9 and S10). No differences in epithelial-to-mesenchymal transition were observed (Supplementary Fig S11). Fully activated hepatic stellate cells isolated from either wild-type or ENKO mice showed similar cell morphology after 8 days in culture (Supplementary Fig S12). However, lentiviral-mediated endosialin knockdown in human immortalized hepatic stellate cells (LX-2 cells) revealed dramatically reduced levels of αSMA and collagens 1 and 6 (Fig 2N) as well as a reduced myofibroblastic phenotype in the shEN HSC compared to control-transfected cells (Fig 2O). While the initiation of liver fibrosis was not altered in ENKO mice, hepatocyte proliferation was significantly increased in ENKO mice compared to WT mice, most notably at the early time points (Fig 3A and B, Supplementary Fig S13), resulting in an increased mean bodyweight after 6 weeks of CCL₄ treatment (Supplementary Fig S14). These findings suggested that the observed ameliorated fibrosis phenotype after 6 weeks might not just have resulted from reduced fibrogenic activity, but rather a shift from a profibrogenic to a proregenerative phenotype in ENKO mice as has previously been observed in 5-hydroxytryptamine 2B receptor (5-HT₂B)-deficient mice (Ebrahimkhani et al., 2011).

Negative regulation of hepatocyte proliferation by HSC-expressed endosialin

We next performed comparative partial hepatectomy (PHx) experiments in WT and ENKO mice in order to examine the role of HSC-expressed EN in a model of rapid hepatocyte proliferation. PHx resulted in a characteristic biophysically upregulated expression of EN (Fig 3C) in HSC with maximum EN expression 1 day after PHx (coinciding with the initiation of hepatocyte proliferation) and 3 days after PHx (coinciding with the initiation of stromal cell proliferation) (Michalopoulos & DeFrances, 1997; Miyaoa et al., 2012). Hepatocyte proliferation was significantly increased in ENKO mice after 2/3 and 1/3 PHx (Fig 3D and E; Supplementary Figs S15 and S16) showing more mitotic figures (Fig 3F), but equal numbers of double nuclei (Supplementary Fig S17). ENKO livers presented with smaller hepatocytes 2 days after hepatectomy, resulting in only slight differences in the liver-to-bodyweight ratio (Supplementary Fig S17). A candidate-based screen of known hepatocyte mitogens identified significantly upregulated levels of IGF2 in livers of ENKO mice (Fig 3G). IGF2 has been proposed to act as a regulator of hepatocyte proliferation (Kimura & Ogihara, 1998), which corresponds to earlier work showing that IGF signaling during liver regeneration affected hepatocyte turnover but not necessarily cell volume (Leu et al., 2003). IGF receptor 1 and IRS1 were activated in ENKO mice. Likewise, phosphorylation of downstream signaling pathways such
as ERK and AKT was upregulated in EN\textsuperscript{KCO} mice (Fig 3H and I). In turn, the expression of other hepatocyte mitogens such as IGF1, HGF, TGF-β1, TGF-β2, TGF-β3, HB-EGF, FGF21, IL-1β, TIMP, or Cdkn1b was not altered (Supplementary Figs S18 and S19). The elevated levels of IGF2 identified in whole liver lysates could not be attributed to a particular cell type in the liver since liver sinusoidal endothelial cells (Fig 3J), hepatocytes (Fig 3K), and HSC (Supplementary Fig S20) were all identified as source of IGF2. Endosialin did not appear to directly affect IGF2 production in HSC as EdU uptake of cultured hepatocytes stimulated with conditioned medium collected from variably activated wild-type or EN\textsuperscript{KCO} HSC was increased to the same extent (Fig 3L). To examine whether IGF2 was able to directly affect hepatocyte proliferation, isolated hepatocytes from both genotypes were stimulated with recombinant IGF2, leading to a significantly increased EdU uptake after 24 h of stimulation (Fig 3M). Together, the selective regulation of IGF2 in livers

**Figure 1. Upregulation of endosialin (EN) expression by activated hepatic stellate cells (HSC) in early liver fibrogenesis.**

A. EN qRT–PCR of murine liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), hepatocytes (Hep), and HSC (n = 3 per cell type).
B. EN qRT–PCR of human hepatocytes (n = 1) and HSC cell line LX-2 (n = 3).
C. EN immunofluorescence staining of LX-2 cells. Scale bar: 25 μm.
D. EN qRT–PCR of normal liver (NL) and fibrotic liver (FL, n = 8 each), and active or inactive cirrhotic liver (CL+/−, n = 10/ n = 2).
E–H. EN immunohistochemistry staining of human normal liver (E), fibrotic liver (F), and cirrhotic liver (G, H). Weak EN expression in portal tracts of normal liver (E). Abundant EN expression detected in activated HSC in fibrous septa of fibrotic (F), active cirrhotic (G), and inactive cirrhotic liver (H). Scale bars: 500 μm.
I–N. Higher magnification of EN-stained normal liver (I), fibrotic liver (K), and (active) cirrhotic liver (M) compared to alpha-smooth muscle actin (αSMA) immunohistochemistry on serial slides (J, L and N). Scale bars: 100 μm.
O. EN/αSMA mRNA ratio in normal liver, fibrotic liver, and active cirrhotic liver determined by qRT–PCR.
P. Collagen 1α (Col1α) expression in normal, fibrotic, and active cirrhotic liver determined by qRT–PCR.

Data information: Data are expressed as mean ± s.e.m.; P-value (significant < 0.05) determined by Student’s t-test.
from ENKO mice suggests a possible role in the observed hepatocyte proliferation phenotype. Yet, further work will be needed to unravel the molecular details of the presumed endosialin–IGF2 axis.

In conclusion, this study has identified the hepatic stellate cell (HSC) marker endosialin (EN) as a critical balance of liver fibrogenesis and regenerative hepatocyte proliferation. Based on three independent pathology models (acute and chronic CCl4-induced liver damage, fibrosis, and partial hepatectomy) and validated by corresponding human pathology specimen expression profiling analyses, EN serves as positive regulator of the fibrogenic stromal cell compartment and as negative regulator of the parenchymal cell hepatocytic compartment (Supplementary Fig S21).

Figure 2. Reduced fibrosis in EN-deficient mice (ENKO) in chronic liver damage. A, B qRT–PCR for EN (A) and αSMA (B) of liver lysates from single high-dose CCl4- or oil-treated wild-type (WT) mice (n = 2 per time point). C Early activated and long-term-activated HSC isolated by differential density gradient centrifugation (8.26% versus 11.5% density layer). qRT–PCR for EN from early activated (8.26%) and long-term-activated HSC (11.5%) from 4-week CCl4-treated mice and vehicle control mice (n = 3–4 per data point). D EN expression in liver lysates from 2-, 4-, and 6-week CCl4-treated mice (n = 5–8) and vehicle control mice (n = 3) determined by qRT–PCR. E–G METAVIR score of 2-, 4-, and 6-week CCl4-treated mice (F–G) and vehicle control WT (F) and ENKO (G) mice. H Mean Col1a+ area per high power field (HPF) shown as percentage of the total area (n = 5–8). I Western blot analysis for collagen 1a (Col1a), platelet-derived growth factor receptor beta (PDGFRβ) and β-actin of total liver protein lysates from 6-week CCl4-treated or control vehicle WT and ENKO mice (n = 3–5). J–L Double immunofluorescence of 6-week CCl4-treated and vehicle control WT (J) and ENKO (K) mice for desmin and αSMA (L) shown as percentage of the total area (n = 5–8). M Serum liver enzymes (GPT/ALT and GOT/AST) from 2-, 4-, and 6-week CCl4-treated WT and ENKO mice (n = 5–8). N qRT–PCR of endosialin (EN), αSMA, and collagen 1a1/6a1 from lentiviral-mediated endosialin knockdown (shEN) and control-transfected (CTL) human hepatic stellate cells (LX-2). O Lentiviral-mediated endosialin knockdown (shEN) and control (CTL) in LX-2 cells (n = 3). Magnification 10×.

Data information: Scale bars: 250 μm. CTL = vehicle control mice. Data are expressed as mean ± s.d. (A–D), or s.e.m. (E, H, L and M); P-value (significant < 0.05) determined by Student’s t-test. Source data are available online for this figure.
Future work will need to unravel the molecular details of the paracrine endosialin-mediated cross talk between HSC and hepatocytes, most notably the role of IGF2 as a regulator of hepatocyte proliferation. Yet, the identification of an HSC-specifically expressed targetable and druggable cell surface receptor that controls the balance between liver fibrosis and liver regeneration may pave the way to fibrosis targeting and at the same time liver regeneration enhancing therapies: The dual compartment functions of endosialin further establish the molecular interdependency of HSC and hepatocyte contribution to liver function in health and disease (Ebrahimkhani et al., 2011). Moreover, EN targeting is presently pursued in early clinical tumor trials. Thus, the data...

Figure 3. Negative regulation of hepatocyte proliferation by HSC-expressed endosialin.
A Representative images of Ki-67 immunohistochemistry after 2, 4, and 6 weeks of CCl₄ treatment of WT and ENKO mice (n = 5–8 mice per group and time point).
B Quantitation of Ki67 hepatocytes/HF after 2, 4, and 6 weeks of CCl₄ treatment.
C Time course of EN expression after partial hepatectomy (2/3 PHx) determined by qRT-PCR of total liver lysates (n = 5/time point, sham: n = 3).
D Representative images of Ki-67 immunohistochemistry after 2/3 PHx of WT and ENKO mice (n = 5–9 mice per group and time point).
E Quantitation of Ki-67 hepatocytes/HF determined 1, 2, 4, and 8 days after 2/3 PHx.
F Number of mitotic figures 2 days after 2/3 PHx of WT and ENKO mice (n = 5–9 mice per group and time point).
G qRT–PCR analysis of total liver lysates 1 day after PHx for IGF2.
H Western blot analysis of phosphorylated insulin-like growth factor receptor 1 (pIGFR1), phosphorylated insulin receptor substrate 1 (pIRS1) and β-actin 2 days after PHx.
I Western blot analysis of phosphorylated and total extracellular signal-regulated kinase (ERK) and AKT 2 days after PHx.
J, K qRT–PCR of IGF2 in isolated liver sinusoidal endothelial cells (LSEC) or hepatocytes (HEP) 1 day after PHx.
L Edu-positive hepatocytes (in %) after 24-h stimulation with conditioned medium (CM) from either WT or ENKO HSC (3, 5, or 7 days of activation). HGF (40 ng/ml) and TGF-β (10 ng/ml) serve as positive or negative control, respectively.
M Edu-positive WT and ENKO hepatocytes (in %) after stimulation with IGF2 (100 ng/ml). HGF (40 or 10 ng/ml) and TGF-β (10 ng/ml) serve as positive or negative control, respectively.

Data information: Scale bars (D, E): 100 μm. Data are expressed as mean ± s.e.m. P-value (significant < 0.05) determined by Student’s t-test.

Source data are available online for this figure.
identify EN as an attractive non-oncologic target for liver fibrosis, whose therapeutic inhibition could negatively impact fibrosis, while at the same time stimulate hepatocyte proliferation.

Materials and Methods

Patient samples

Tissue samples were provided by the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the ethics committee of the University of Heidelberg. This study was performed according to the Declaration of Helsinki; written informed consent was obtained from all patients. All patient specimens and corresponding clinical information were exclusively provided in a pseudonymized form according to the standard operating procedures of the NCT, approved by the ethic committee of the University of Heidelberg (Ethikvotes #206/207, 2005). Primary human hepatocytes were kindly provided by PD Dr. K. Breuhahn (Institute of Pathology, Heidelberg).

Animal experiments

All animal experiments were performed according to the guidelines of the local animal use and care committees and approved by the Regierungspräsidium in Karlsruhe, including animal permits G195/10 (CCl4 experiments) and G220/11 (partial hepatectomy). Animals were housed in barriers at the animal facility of the DKFZ with free admission to food and water. For sacrificing, mice were anesthetized by intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg) diluted in isotonic 0.9% NaCl. Deeply anesthetized mice were heart punctured to obtain blood followed by rapid dislocation of the cervical spine. Whole blood was centrifuged at 8,000 g for 10 min, and subsequently, serum was collected from the supernatant. For determining clinically relevant serum parameters, 300 μl of serum (if necessary diluted with isotonic 0.9% NaCl) was analyzed by the Diagnostic Center of the Heidelberg University Hospital.

To induce acute liver damage, C57/B6 WT mice at an age of 10 weeks were injected intraperitoneally with a single high dose of CCl4 (1.6 g/kg CCl4, diluted in mineral oil). Mice were sacrificed after 3, 6, 24, 48, 72 h, or 6 days after injection. CCl4-induced liver fibrosis experiments were performed as previously described (Constandinou et al., 2005). Five to seven male C57/B6 endosialin WT and KO mice group at an age between 10 and 15 weeks were used for these experiments. Mice were injected intraperitoneally with CCl4 (1 ml/kg bodyweight, 1:7 dilution in olive oil) twice a week for 2, 4, or 6 weeks. Control mice received the same amount of olive oil as CCl4-treated animals during the experimental time. Mice were sacrificed 3 days after the last CCl4 injection. Partial hepatectomy was performed as previously described (Mitchell & Willenbring, 2008). In case of performing 1/3 partial hepatectomy, only left lobe was removed. Five to ten male C57Bl/6 endosialin WT and KO mice group at an age between 10 and 15 weeks were used for partial hepatectomy experiments. Mice were anaesthetized by intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg) diluted in isotonic 0.9% NaCl. As control, mice were sham-operated. Mice were sacrificed after 1, 1.5, 2, 4, or 8 days posthepatectomy.

Statistical analysis

All results are expressed as mean ± s.e.m. or s.d., as indicated. The statistical differences between WT and ENKO mice groups were analyzed using the two-tailed unpaired Student’s t-test. The statistical difference between different time points in WT mice was analyzed using the two-tailed paired or unpaired t-test. Correlations were analyzed using the Pearson (bivariate) correlation. Differences P ≤ 0.05 were considered statistically significant.

Additional materials and methods

Detailed information on additional materials and methods related to cells and cell culture experiments, molecular techniques, biochemical protocols and reagents, and histological and immunohistological techniques are summarized in the Supplementary Information.
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Author contributions
All authors have read and approved the manuscript. CM performed human data analysis, human and murine histology evaluation, data analysis, and interpretation and contributed to the writing of the manuscript. MW performed mouse experiments, cells isolations, data analysis, and interpretation and contributed to the writing of the manuscript. CK performed mouse experiments, data analysis, and interpretation. JH, AR, CK, and EB performed mouse experiments, cell isolations, data analysis, and interpretation. KBH and PS performed human data analysis and histology. HGA performed experimental outline of the study, data analysis, and interpretation and contributed to the writing of the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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