Hepatic stellate cells limit hepatocellular carcinoma progression through the orphan receptor endosialin

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common and deadliest cancers worldwide. A major contributor to HCC progression is the cross talk between tumor cells and the surrounding stroma including activated hepatic stellate cells (HSC). Activation of HSC during liver damage leads to upregulation of the orphan receptor endosialin (CD248), which contributes to regulating the balance of liver regeneration and fibrosis. Based on the established role of endosialin in regulating HSC/hepatocyte cross talk, we hypothesized that HSC-expressed endosialin might similarly affect cell proliferation during hepatocarcinogenesis. Indeed, the histological analysis of human HCC samples revealed an inverse correlation between tumor cell proliferation and stromal endosialin expression. Correspondingly, global genetic inactivation of endosialin resulted in accelerated tumor growth in an inducible mouse HCC model. A candidate-based screen of tumor lysates and differential protein arrays of cultured HSC identified several established hepatotropic cytokines, including IGF2, RBP4, DKK1, and CCL5 as being negatively regulated by endosialin. Taken together, the experiments identify endosialin-expressing HSC as a negative regulator of HCC progression.

Keywords cancer; HCC; stromal cross talk; tumor stroma; vascular biology

Subject Categories Cancer; Digestive System

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadliest cancers worldwide with hundreds of thousands of deaths each year (746,000 reported deaths in 2012; Londono et al., 2015; Thompson et al., 2015). Most HCC develop as a result of chronic liver damage strongly depending on the cross talk of hepatocytes and the stromal microenvironment, which may foster a pro-inflammatory and pro-tumorigenic milieu (Coulouarn et al., 2012). The role of tumor stroma has long been shown to promote tumor growth and invasiveness either via direct cell-cell interaction, through the secretion of tumor-promoting cytokines such as hepatocyte growth factor (HGF) and transforming growth factor beta (TGFβ), or by modulating extracellular matrix components via integrins or fibroblast growth factors (Liu et al., 2011; Desert et al., 2016; Aflo et al., 2017). However, recent data also suggest that the tumor stroma may exert protective anti-tumorigenic functions (Bissell & Hines, 2011; Dittmer & Leyh, 2015).

The tumor stroma consists of several cell types, including endothelial cells, macrophages, and hepatic stellate cells (HSC; Heindryckx & Gerwins, 2015). HSC and other tumor-associated mesenchymal-derived cells such as myofibroblasts and pericytes express the orphan receptor endosialin (CD248; MacFadyen et al., 2005, 2007; Christian et al., 2008; Simonavicius et al., 2008; Mogler et al., 2015). As a marker of the activated mesenchymal lineage, endosialin plays a critical role in the development and progression of liver, kidney, and pulmonary fibrosis (Chang-Panesso & Humphreys, 2015; Mogler et al., 2015; Barts et al., 2016). In turn, endosialin negatively regulates hepatocyte proliferation, thereby balancing the epithelial and the stromal response after acute and chronic liver damage (Mogler et al., 2015).

Endosialin is only weakly expressed in healthy adult tissues, but prominently upregulated in the stromal compartment of...
progressing tumors. Genetic inactivation or antibody-mediated inhibition of endosialin resulted in reduced primary tumor growth and metastasis in mouse models of colon cancer, melanoma, and breast cancer (Nanda et al., 2006; Rybinski et al., 2015; Viski et al., 2016). Endosialin therefore may be an attractive target for stromal-based therapeutic approaches in malignancies. An endosialin-blocking antibody (MORAb-004) is in early clinical development and appears to exert some clinical efficacy in a small cohort of patients with different solid extra-cerebral malignant tumors (including carcinoma, sarcoma, and neuroendocrine tumors; Diaz et al., 2015). We have previously observed that HSC-expressed endosialin promotes liver fibrosis and in turn negatively regulates hepatocyte proliferation, thereby acting as a balance of fibrosis vs. regeneration (Mogler et al., 2015). Based on these findings, the present study was aimed at studying the role of endosialin during HCC progression.

Results and Discussion

Endosialin is heterogeneously expressed in human HCC and inversely correlates with tumor cell proliferation

To assess the expression of endosialin during HCC progression, we performed immunohistochemical analyses in whole tissue HCC samples of different stage. Consistent with our previous findings (Mogler et al., 2015), expression of endosialin in healthy liver was weakly detectable exclusively in hepatic stellate cells (HSC) and portal myofibroblasts (Fig 1A). Endosialin was strongly upregulated along the fibrous septa and to lesser extent also along the sinusoids in liver cirrhosis (Fig 1B; Mogler et al., 2015). In dysplastic nodules (DN, n = 5), the premalignant lesions of HCC, endosialin was focally expressed in the sinusoidal compartment surrounding dysplastic hepatocytes (Fig 1C and Appendix Fig S1).

Figure 1. Endosialin is heterogeneously expressed in human HCC and stromal-expressed endosialin inversely correlates with tumor cell proliferation.

A–D: Endosialin immunohistochemistry staining of whole tissue slides of human healthy liver (A) (n = 5), cirrhotic liver (B) (n = 10), dysplastic nodules (DN, C) (n = 5), and hepatocellular carcinoma (HCC, D) (n = 13). Weak endosialin expression in portal tracts of normal liver (A), stronger expression of endosialin in the cirrhotic liver (B), along the sinusoids of dysplastic nodules (C), and within the HCC stroma (D).

E, F: Heterogeneous distribution of stromal-expressed endosialin.

G, H: Intense expression of endosialin at the fibrous capsule/invasion front of HCC (H shows a close-up of G; CL = nodules of cirrhotic liver).

I, J: Immunohistochemical double stains of endosialin (red) and the proliferation marker Ki67 (brown) in HCC.

K: Correlation of overall intratumoral endosialin expression and HCC tumor cell proliferation.

Data information: Scale bars: as indicated. Arrows indicate positive endosialin staining. Statistical analysis: Pearson’s correlation, r = correlation coefficient.
In human hepatocellular carcinoma, endosialin was detectable with considerable regional variation in the stroma of all specimens with most abundant expression at the invasion front and in the fibrous capsule surrounding tumor cells (n = 13; Fig 1D–H). Correspondingly, when analyzing endosialin expression in tissue microarrays, only 31.5% of HCC punches (n = 57) showed detectable endosialin expression (Appendix Fig S2). Yet, in both, whole slides and TMA punches, endosialin expression was restricted to mesenchymal cells within the tumor (predominantly stellate cells and [myo]-fibroblasts) and tumor cells were consistently negative for endosialin. To rule out an expression of endosialin by tumor-associated endothelial cells [as proposed earlier by St Croix et al (2000)], double immunohistochemical stainings of CD31 and endosialin were performed confirming that endosialin in HCC was exclusively expressed by non-endothelial mesenchymal cells (Appendix Fig S3). No correlation between endosialin expression and the underlying etiology of the HCC samples was found (including viral hepatitis [n = 5], ASH [n = 4], NASH [n = 4]). However, when regionally quantitating tumor cell proliferation (by Ki67 immunohistochemistry of whole tissues slides), the abundance of stromal endosialin-expressing cells was inversely correlated with HCC tumor cell proliferation (Fig 11–K).

Enhanced HCC tumorigenesis in endosialin-deficient mice

To study the role of endosialin in an experimental model of HCC progression, we bred WT and endosialin-deficient mice (ENKO) with mice expressing the polyoma middle T antigen Cre-inducible under the control of the albumin promoter (iAST mouse model; Runge et al., 2014). ENKO::iAST mice were viable and did not display any overt phenotype in unchallenged settings (Appendix Fig S4). Tumorigenesis in livers of WT::iAST and in ENKO::iAST mice was induced by tail vein injection of Cre-expressing adenovirus, and tumor growth in WT::iAST and ENKO::iAST mice was monitored non-invasively by weekly computed tomography (CT) scans (Fig 2A and B, and Appendix Fig S5). ENKO::iAST presented significantly more CT-detectable tumors 6 weeks after tumor induction (Fig 2C). Tumors were harvested 8 weeks after induction, when ENKO::iAST mice presented macroscopically significantly more tumor nodules (Fig 2D–F), higher total liver weight (Fig EV1), histologically increased tumor burden (Fig 2G–I), and elevated tumor cell proliferation assessed by immunohistochemistry and Western blot analysis of the proliferation markers Ki67 (Fig 2J–L) and PCNA (Figs 2M–O and EV2). Histological analysis of samples harvested at earlier time points (4 weeks after tumor induction) revealed the same phenotype (Fig EV3).

Endosialin silencing reduces HSC proliferation and enhances HCC tumor cell proliferation

We next performed cell culture experiments of HSC monolayers and HSC-HCC tumor cell co-cultures to yield mechanistic insight into the observed human and mouse phenotypes (Fig 3). Lentivirally endosialin-silenced immortalized human HSC (shEN) displayed an altered morphology with less myofibroblast-typical morphology compared to non-silenced (nsEN) control HSC (Fig 3A). Proliferation of shEN cells was strongly reduced compared to nsEN cells (Fig 3B). Stimulation of HSC by co-culture with human HCC tumor cells (Huh7) did not rescue the reduced proliferation of shEN cells (Fig 3C and D).

To study paracrine effects of HSC on HCC cells, we stimulated Huh7 cells with conditioned medium (CM) from shEN cells, which led to increased tumor cell proliferation compared to stimulation with CM from nsEN (Fig 3E). Previous experiments had identified insulin-like growth factor-2 (IGF-2) as a putative HSC-derived hepatocyte mitogen (Mogler et al., 2015) and a contributor to hepatocarcinogenesis (Tovar et al., 2010). Correspondingly, differential expression profiling experiments of shEN and nsEN LX-2 cells as well as of tumor lysates from WT::iAST and ENKO::iAST mice revealed a significant upregulation of IGF-2 in shEN cells and in whole liver lysates of ENKO::iAST mice (Fig 3F and G). Correspondingly, silencing the primary IGF-2 receptor, insulin-like growth factor receptor 1 (IGFR1), in cultured Huh7 cells, resulted in a significant reduction of tumor cell proliferation (Fig 3H). Albeit not formally establishing a causal relationship, the data support the hypothesis that endosialin regulates IGF-2 expression in HSC, which in a paracrine manner controls HCC tumor cell proliferation.

To test, if other paracrine factors beyond IGF-2 may contribute to the paracrine cross talk between HSC and HCC cells, we performed additional cytokine array experiments of CM from endosialin-silenced and non-silenced HSC (Appendix Fig S6A).
Figure 2.

CT detectable nodules [average/group]

|       | WT: iAST | EN\textsuperscript{KO}: iAST |
|-------|----------|-----------------------------|
| 6 weeks |   0.019  |                             |
| 7 weeks |   0.020  |                             |

Detectable nodules [n]

|       | WT: iAST | EN\textsuperscript{KO}: iAST |
|-------|----------|-----------------------------|
|       |   25.0   |                             |

Tumor/normal [%]

|       | WT: iAST | EN\textsuperscript{KO}: iAST |
|-------|----------|-----------------------------|
|       |   40.0   |                             |

Ki67+ hepatocytes [%]

|       | WT: iAST | EN\textsuperscript{KO}: iAST |
|-------|----------|-----------------------------|
|       |   50.0   |                             |

PCNA+ hepatocytes [%]

|       | WT: iAST | EN\textsuperscript{KO}: iAST |
|-------|----------|-----------------------------|
|       |   30.0   |                             |

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Figure 3.
These experiments identified a number of established HCC-related cytokines as being produced by HSC in an endosialin-dependent manner (see Appendix Table S1 for complete list of cytokines). Among the most strongly upregulated molecules expressed by endosialin-silenced hepatic stellate cells was the chemokine (C-C motif) ligand 5 (CCL5; Bai et al., 2014), retinol-binding-protein 4 (RBP4; Wang et al., 2011), Dickkopf-1 (DKK1; Ge et al., 2015), platelet-derived growth factor-α (PDGF-AA; Wei et al., 2014), and urokinase-type plasminogen activator receptor (uPAR; Zheng et al., 2014), all well-known promoters of HCC progression and aggressiveness. Huh7 cells stimulated with either CCL5 or RBP4 indeed showed higher proliferation compared to untreated tumor cells indicating that IGF2 might be a major, but not the only endosialin-controlled HSC secreted factor affecting HCC cells in a paracrine manner (Appendix Fig S6B and C). Together, these data provide strong evidence that hepatic stellate cell-expressed endosialin plays an important role in negatively controlling tumor and positively controlling stromal cell proliferation in the setting of hepatocarcinogenesis involving complex autocrine and paracrine mechanisms (Fig 3I).

The findings of the present study shed further insight into the complexity of tumor cell-stromal cell cross talk during hepatocellular carcinogenesis. Previous work on the pathogenesis of HCC has primarily focused on the molecular mechanisms governing neoplastic parenchymal cell transformation. Thereby the contribution of stromal cells to HCC initiation and progression may have been underestimated (Thompson et al., 2015). Among the stromal cell types, HSC have been most extensively studied for their role in regulating liver function in a paracrine manner, for example, during regeneration, fibrosis and cirrhosis from which approx. 90% of HCC develop (Forner et al., 2012). Yet, the mechanisms of HSC contribution to HCC represent a complex, to date poorly understood role in tumorigenesis. Intriguing findings are highly contradictory implicating a “good” vs. “bad” phenotype of HSC. For example, the activation and associated phenotype of HSC have been shown to contribute to HCC development and progression by secreting proliferation-inducing cytokines, such as hepatocyte growth factor (HGF; Matsumoto & Nakamura, 2006) or by initiating in a paracrine manner tumor angiogenesis (Zhu et al., 2015). In turn, activated HSC are a source of transforming growth factor beta (TGFβ), which exerts growth inhibitory effects, particularly in early tumor stages (Meindl-Beinker et al., 2012). These apparently discrepant findings may point toward context-dependent pro- and anti-tumorigenic effects of HCC-associated HSC. In the present study, we could show in definite genetic settings that endosialin, which is in the liver expressed by HSC and stromal myofibroblasts, acts as a negative regulator of HCC. Moreover, albeit restricted to a limited cohort of samples, there was a strong inverse correlation between tumor cell proliferation and stromal endosialin expression in human HCC suggesting that the paracrine cross talk observed in the preclinical mouse model may also exist in human HCC. The loss of HSC-expressed endosialin led to an increase of tumor-promoting secreted factors, including IGF-2 and CCL5.

The findings of this study also contribute to the increasing appreciation that stromal cells do not just exert pro-tumorigenic functionalities, but may restrain tumor growth. For example, preclinical pancreatic ductal adenocarcinoma experiments have suggested that the targeting of tumor-associated fibroblasts may stimulate rather than inhibit tumor growth (Ozdemir et al., 2014; Rhim et al., 2014). These landmark studies have inspired the concept that stromal reprogramming may be therapeutically exploited to balance tumor cell proliferation (Rowley, 2014). Our findings expand these concepts by ascribing a distinct cell surface receptor, the orphan receptor endosialin (CD248), to the paracrine tumor growth regulating properties of HCC-associated hepatic stellate cells. Future work will need to focus on the mechanisms controlling the activation status of HSC that determines the phenotypic switch from “good” to “bad” (or vice versa?). As shown here, while endosialin-expressing and fully activated HSC impair tumor growth, endosialin-deficient, partially activated HSC promote HCC progression. Taken together, this study establishes a causal inverse relationship between hepatic stellate cell-expressed endosialin and growth of HCC and put a cautionary note on the potential application of endosialin-blocking antibodies in HCC.

Materials and Methods

Patient samples

This study was registered at the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) and performed according to the Declaration of Helsinki; written informed consent was obtained from all patients. All patient specimens were exclusively provided in a pseudonymized form.
according to the Standard Operating Procedures of the NCT, approved by the Ethics Committee of the Heidelberg University (Ethical votes # 206/207, 2005).

**Animals**

All animal experiments were performed according to the guidelines of the local Animal Use and Care Committees and approved by the Regierungspräsidium in Karlsruhe (35-9185.81/G-228/10). Animals of the local Animal Use and Care Committees and approved by the All animal experiments were performed according to the guidelines (Ethical votes # 206/207, 2005).

**Computer tomography (CT)**

Mice were anaesthetized using isoflurane (1.5%) and oxygen (0.5 l/min). Respiratory-gated volumetric computed tomograph (VCT) imaging was done on a prototype flat-panel equipped volumetric computed tomograph (Volume CT, Siemens; tube voltage, 80 kV; tube current, 50 mA; scan time, 80 s; frames per second, 120; rotation speed, 10 s; Kernel H80a; matrix 512 × 512. Fenestra LC contrast agent (100 μl; Vector Biolabs) was diluted to 1 × 10^6 PFU/100 μl with isotonic 0.9% NaCl and injected into the tail vein.

**Processing of formalin-fixed paraffin-embedded and cryo-sectioned tissues**

All organs and tissues were fixed in 4% PFA overnight, processed and stained (H&E) according to standard procedures. For cryofixation, tissues were placed in OTC cryo-medium, or alternatively on cork plates, and were snap-frozen in liquid nitrogen or on dry ice.

**Immunohistochemistry stainings on paraffin sections**

Paraffin sections were dewaxed according to standard procedures. For antigen-retrieval slides were boiled for 20 min in 0.01 M pH 6.0 citrate target retrieval buffer and treated with 3% H2O2 for 15 min to block endogenous peroxidase. Slides were incubated with primary antibodies [endosialin: kindly provided by C. Isacke 1:500; Ki67 (human): 1:100 (Dako, Hamburg, Germany); Ki67 (mouse): 1:50 (Dako, Hamburg, Germany), PCNA: 1:500 (My Biosource, San Diego, USA)]. Detection was performed via a biotin – peroxidase complex according to manufacturer’s protocol. Sections were counterstained with hematoxylin.

**Quantitative real-time PCR (qRT–PCR)**

DNA from cells or pieces of liver was purified by the Rneasy Mini Kit from Qiagen according to manufacturer’s instructions. Concentration and purity were measured on the Nanodrop (Eppendorf). cDNA was generated using the Quantitect® Reverse Transcription Kit (Qiaegen) according to manufacturer’s instructions. Quantitative real-time RT–PCR (qRT–PCR) was performed on an ABI StepOne-Plus cycler using TaqMan probe sets purchased from Applied Biosystems.

**Western blot analysis**

Protein lysates were prepared from frozen organs or cryo-material. Liver material was lysed in NP-40 lysis buffer with phosphatase inhibitor orthovanadate (2 mM). Protein concentrations were determined by Bradford or the BCA assay. Proteins were separated on 10% polyacrylamide/SDS gels, blotted on nitrocellulose membranes, and incubated with primary antibodies at 4°C [PCNA: 1:500 (My Biosource, San Diego, USA)] overnight. Detection of chemiluminescence was done with ECL Western blotting substrate (Pierce). Scanning and evaluation was performed using the Amersham Western Blotting System (GE Healthcare, Munich, Germany).

**Cells and SH mediated lentiviral knockdown**

The human HSC cells LX-2 were purchased from Millipore (Darmstadt, Germany). LX-2 were cultured in DMEM 1 g glucose, 2% fetal calf serum, and 1% penicillin/streptomycin (PS), at 37°C, 5% CO2. Cells were passaged as necessary. Endosialin knockdown was achieved by GFP-labeled lentiviral-mediated transfection of LX-2 cells using either non-silencing (nsEN) or endosialin-silencing (shEN) virus as already published (Mogler et al, 2015). Silencing of gene expression was validated by qPCR and was consistently above 90%. Huh7 cells were kindly provided from PD Dr. K. Breuhahn (Institute of Pathology, Heidelberg) and cultured in DMEM (4.5 g glucose, 10% FCS, 1% PS).

**MTT assay**

Of 2,000 LX-2 cells (nsEN and shEN) were seeded in triplicates into 96-well plates (T = 0, 24, 48 h). Assay was performed according to manufacturer’s protocol (Roche, Basel, Switzerland).

**siRNA transfection**

Of 70,000 Huh7 cells were plated in 6-well plates. Solution A (10 μl siNTC (Ambion) or siIGF1R_ds1 (s=1) or siIGF1R_ds3 (s=2; Eurofins Genomics) in 100 μl OptiMEM (Gibco) was incubated for 10 min at RT. Solution B (5 μl Oligofectamine (Invitrogen) in 100 μl OptiMEM, incubated 10 min at RT) was mixed with Solution A and incubated for 30 min at RT. The siRNA mix (A + B) was added dropwise to the cells and incubated for 4 h at 37°C. Knockdown was checked after 48 h by qRT–PCR. IGF1R ds1 (GGACUCAGUACGCCGUUUA)TT IGF1R ds3 (GGCCAGAAAUGGAGAAUAA)TT

**EdU assay**

EdU assay was used for both Huh7 and HSC cells (60,000 Huh7 and 40,000 LX-2/well with three wells per experimental condition). Cells were cultured in 2% FCS at least 24 h prior to experimental procedure. EdU intake and staining was performed using the Click-IT®
EdU Alexa Fluor® 647 Imaging Kit according to the manufacturer’s protocol (Thermo Fisher Scientific, Munich, Germany). Ten random pictures of each well and condition were taken. Pictures were analyzed by two independent investigators (C.M. and C.K.) using ImageJ Software (FIJI).

For stimulation experiments, cytokines (CCL5 and RBP4) were added to the medium (10 ng/ml for CCL5 and 50 ng/ml for RBP4) for 24 h.

**EdU FACS (co-cultures Huh7 and Lx-2)**

60,000 Huh7 and 40,000 Lx-2 (± shEN) cells were incubated for 48 h prior to EdU (1:1,000) incubation for 3 h. EdU staining was performed according to manufacturer’s protocol (Thermo Fisher Scientific). Prior to FACS analysis, cells were stained with FxCycle and resuspended in 100 μl PBS.

**Cytokine array**

Human XL cytokine array was purchased from R&D, and level of cytokines was assessed as per directions of the manufacturer. Briefly, the array membranes were blocked with blocking buffer at room temperature and incubated with supernatant derived from co-cultures (Huh7 and Lx-2 ± shEN) overnight. Detection antibody cocktail was added to the membranes. After incubation and washing, streptavidin-HRP was added to each membrane. After incubation and washing, the cytokines were detected by chemiluminescence reaction. Spots were quantified using FIJI software after background subtraction. Normalized data were analyzed for endosialin knockdown-specific results and used to determine the mean differences in cytokine abundance.

**Statistics**

All results are expressed as mean ± SD. Differences between WT::iAST and ENKO::iAST 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.

**Expanded View** for this article is available online.

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**Author contributions**

CM, CK, and MW designed research; CM, CK, MW, AR, EB, and DK performed experiments; CM, CK, MW, AR, DK, TL, and PS analyzed data; and CM and HGA wrote the manuscript. All authors read and approved the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

The paper explained

**Problem**

We have previously shown that hepatic stellate cells (HSC) promote hepatocyte proliferation during liver regeneration through the orphan receptor endosialin (CD248). We hypothesized that a growth promoting effect during tissue regeneration could possibly translate into a tumor-promoting effect in hepatocellular carcinoma (HCC), one of the deadliest cancers worldwide. Employing a genetic model of endosialin deficiency and a genetic model of HCC tumorigenesis, we studied the role of endosialin during the growth of HCC.

**Results**

Genetic inactivation of endosialin resulted in accelerated tumor growth in an inducible mouse model of HCC. Several hepatotrophic cytokines, including IGF2, RBP4, Dkk1, and CCL5, very identified as being negatively regulated by endosialin. Histological analysis of human HCC samples revealed an inverse correlation between tumor cell proliferation and stromal endosialin expression.

**Impact**

The experiments demonstrate that HCC-associated hepatic stellate cells are capable to inhibit tumor growth and progression through the orphan receptor endosialin. The data contribute to the emerging theme that stromal cells do not just promote tumor growth, but may in fact be part of the host’s defense aimed at restraining tumor growth.

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