Tumor and Stem Cell Biology

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Abstract

The limited availability of experimental tumor models that faithfully mimic the progression of human tumors and their response to therapy remains a major bottleneck to the clinical translation and application of novel therapeutic principles. To address this challenge in hepatocellular carcinoma (HCC), one of the deadliest and most common cancers in the world, we developed and validated an inducible model of hepatocarcinogenesis in adult mice. Tumorigenesis was triggered by intravenous adenoviral delivery of Cre recombinase in transgenic mice expressing the hepatocyte-specific albumin promoter, a loxP-flanked stop cassette, and the SV40 large T-antigen (iAST). Cre recombinase–mediated excision of the stop cassette led to a transient viral hepatitis and resulted in multinodular tumorigenesis within 5 to 8 weeks. Tumor nodules with histologic characteristics of human HCC established a functional vasculature by cooptation, remodeling, and angiogenic expansion of the preexisting sinusoidal liver vasculature with increasing signs of vascular immaturity during tumor progression. Treatment of mice with sorafenib rapidly resulted in the induction of vascular regression, inhibition of tumor growth, and enhanced overall survival. Vascular regression was characterized by loss of endothelial cells leaving behind avascular type IV collagen–positive empty sleeves with remaining pericytes. Sorafenib treatment led to transcriptional changes of Igf1, Id1, and cMet over time, which may reflect the emergence of potential escape mechanisms. Taken together, our results established the iAST model of inducible hepatocarcinogenesis as a robust and versatile preclinical model to study HCC progression and validate novel therapies. Cancer Res; 74(15): 4157–69. ©2014 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in woman, and overall the third leading cause of death from cancer worldwide (1). It commonly develops from cirrhotic livers due to chronic hepatitis B and C infections (2,3) or a history of alcohol consumption. Multifocal intrahepatic tumor development is common (4) and the prognosis is poor due to a limited response to systemic chemotherapy and radiotherapy, and lack of other treatment options. Early diagnosed patients are eligible for tumor resection or transplantation if cirrhosis is present. However, the incidence of tumor recurrence is very high. A widely accepted technique to treat HCC is transarterial chemoembolization, during which tumor-associated arterial vessels are selectively obstructed, resulting in tumor necrosis (5).

Recently, sorafenib, a small multikinase inhibitor, has shown some efficacy as monotherapy in patients with late-stage HCC (6). Yet, the overall clinical benefit of sorafenib in terms of time-to-progression and overall survival (OS) is rather modest (7), highlighting the need to mechanistically better understand beyond the molecular target specificity how sorafenib is actually interfering with tumor growth and what limits its clinical efficacy. As a multikinase inhibitor targeting the Raf/MEK/ MAPK, VEGFR, and PDGFRβ signaling pathways (8), sorafenib exerts direct antitumorigenic as well as antiangiogenic effects, which have been demonstrated in various animal models as well as in patients (9,10). Yet, the detailed functional mechanism of action in vivo has not been analyzed in much detail.

The better mechanistic understanding of the effects of sorafenib on HCC progression is also of general interest.
because angiogenesis in HCC is different from angiogenesis in other solid tumors as a consequence of the unique vascular properties of the liver. The intrabiliary vasculature consists of a dual vascular system, the arterial/venous, and the sinusoidal endothelium. Both are believed to contribute to HCC development by changing from a venous towards a capillary endothelial cell-like phenotype with expression of markers of continuous, nonfenestrated endothelial cells (11). Indeed, early intratumoral blood supply appears to primarily depend on the sinusoidal vasculature with later involvement of the hepatic arterial system (12, 13). HCC angiogenesis depends on the activation, proliferation, and migration of endothelial cells as well as of pericytes, events that are also found in other tumors with high angiogenic potential (14). Clinical studies have solidly established a correlation of the angiogenic activity with a high risk of vascular invasion, metastasis, and poor prognosis (15, 16).

A number of animal models have been established for the study of HCC progression. These include subcutaneous and orthotopic grafting models, genetically engineered mouse models, as well as chemically induced carcinogenesis models (17). While all of these models resemble key features of the pathogenesis of HCC, many of them are also somewhat limited by either the large global induction of tumorigenesis in the liver, which hardly mimics the multinodular development of liver, which hardly mimics the multinodular development of HCC as it occurs in humans, or the long duration until tumor development, which in some of the genetic models might take up to 2 years (18). Likewise, none of the established models has been optimized for the analysis of HCC angiogenesis, which is one of the reasons for the hitherto poorly characterized effects of sorafenib in experimental models of HCC.

The Riptag model of endocrine pancreas tumorigenesis (Riptag = rat insulin promoter – SV40 large T-antigen) has over the years proven to be a powerful system for the spatiotemporal analysis of intratumoral angiogenesis during tumor initiation and tumor progression (19, 20). SV40 large T-antigen–driven tumorigenesis has also been employed for the induction of liver tumorigenesis (21). While global constitutive liver Tag induction may yield limited insights into HCC pathogenesis, inducible models of Tag-driven hepatocarcinogenesis may be particularly suitable to mechanistically dissect the intricate tumor–stromal crosstalk during HCC progression, including the induction of angiogenesis. We have, in the present study, taken advantage of a recently developed inducible variant of Tag-driven hepatocarcinogenesis, in which tumorigenesis is induced through the adenoviral delivery of Cre recombinase (22). The unprecedented versatility, robustness, and temporal properties of this model provide unique opportunities to spatiotemporally study the progression of HCC and the response of HCC to therapy.

Materials and Methods

Animals and tumor induction
Cre-inducible AST transgenic mice (22) bred in the C57/Bl6 background were housed in the institutional animal facility. Neoplastic transformation was induced in 8- to 12-week-old mice by tail-vein injection of 10⁹ infectious units of adenovirus expressing Cre recombinase (Vector BioLabs). Genotype of the transgene was confirmed by PCR. All experiments were approved by the institutional and governmental Animal Care and Use Committees [RP Karlsruhe (35-9185.81/G-228/10)].

In vivo studies
Mice were treated daily with 20, 40, 60, or 100 mg/kg of sorafenib delivered by oral gavage. Sorafenib, p-Toluenesulfonate (LC Laboratories), was diluted in 1:1 Cremophor EL (Sigma)/50% ethanol. Vehicle-treated animals received 1:1 Cremophor EL/50% ethanol solution. For perfusion experiments, 150 μL of 1 mg/mL lectin (FITC-labeled Bandeiraea simplicifolia; Sigma) for leukocyte analysis or 150 μL of 60 mg/kg dextran (TRITC-Dextran, low molecular weight: 65–85 kDa, Sigma) and for hypoxia, 150 μL of 60 mg/kg pimonidazole was injected into the tail vein 20 minutes before sacrifice.

Computed tomography
Mice were anesthetized using a mixture of isoflurane (1.5%) and oxygen (0.5 L/min). Hundred microliters of Fenestra LC contrast agent (LC-133; Advanced Research Technologies, Inc.) was injected intravenously in the tail vein. Respiratory-gated VCT imaging was performed on a prototype flat panel–equipped volumetric computer tomograph (Volume CT, Siemens) with the following parameters: tube voltage, 80 kV; tube current, 50 mA; scan time, 80 seconds; frames per second, 120; rotation speed, 10 seconds; Kernel H80a; matrix 512 × 512. Images were processed with the program OsirIX v.5.5.1 (23). The program was used with GPU engine and active shading at default settings. Image series were imported and processed with the function “3d volume rendering.”

Magnetic resonance imaging
Mice were anesthetized using a mixture of isoflurane (1.5%) and oxygen (0.5 L/min). Magnetic resonance images were acquired on a 1.5-T clinical MR scanner (Symphony, Siemens). A home-built coil for radiofrequency excitation and detection was used and designed as a cylindrical volume resonator with an inner diameter of 83 mm and a usable length of 120 mm. T₂-weighted images were acquired using a turbo spin-echo sequence (orientation axial, TR 3,240 ms; TE 81 ms; matrix, 152 × 256; resolution, 0.35 × 0.35 × 1.5 mm³; 3 averages, 15 images; scan time, 3:40 min). Images were processed with The Medical Imaging Interaction Toolkit (MITK version 0.10.0, 2008, freeware). Image series were imported and tumor nodules were marked using the “interactive segmentation” tool. Subsequently, nodule volume was determined in ml using the volumetry function of the same tool. The five largest nodules were analyzed to assess tumor progression over time. Tumor volumes were averaged per mouse and per time from vehicle (n = 8) and sorafenib (n = 9) treated animals, respectively. Values were grouped in 3 time points starting with the treatment initiation (week 0) and the respective follow-up (week 1 and week 2).

Immunohistochemistry
Paraffin-embedded liver samples were dehydrated followed by antigen retrieval using citrate buffer, pH 6.0 (DAKO). Before
antibody incubation, slides were subjected to 3% H₂O₂ to inactivate endogenous peroxidase activity and blocked in 4% BSA/2% normal goat serum diluted in 1× TBS/Tween-20 (0.5%; AppliChem). The following primary antibodies were used: anti-mouse glypican 3 antibody (1:200; Abcam); rabbit anti-mouse HSP70 (1:100; Cell Signaling Technology); polyclonal rabbit anti-human CD3 antibody (1:300, Dako); polyclonal rabbit anti-IGF1R antibody (1:300, Cell Signaling Technology); polyclonal goat anti-mouse VEGF164 antibody (1:10; R&D Systems); and secondary goat anti-rabbit or goat anti-rat IgG biotinylated antibody (1:200, Vector Laboratories), or donkey anti-goat IgG biotinylated antibody (1:200, Jackson Dianova). Antibody detection was visualized by 3,3′-diaminobenzidine after ABC-HRP incubation and sections were counterstained with Mayer’s hematoxylin. Hypoxia was assessed using Hypoxyprobe-1 Plus Kit (hpi) including FITC-conjugated mouse IgG (1:50) and rabbit HRP-conjugated anti-FITC secondary antibody (1:100); antigen retrieval was performed in Citric-EDTA buffer. Human HCC nodules were detected with two different mouse monoclonal antibodies: anti-human Glypican 3 antibody Clone 1G12 (1:100; Biomabs); anti-human HSP70 antibody (1:250; Santa Cruz); and rabbit-anti-mouse biotinylated IgG (1:250 Dako). Staining was performed using an automated immunohistochemical stainer (Techmate 500, Dako Cytomation) and ChemMate detection kit in accordance with the manufacturer’s protocol.

Cryosections. Cryosections (7 μm) were methanol-fixed and the tumor vasculature was detected by rat anti-mouse CD31 staining (1:100; BD Pharmingen) and goat anti-rat IgG conjugated with Alexa 488 (1:500; Invitrogen). Tumor pericytes were detected with two different antibodies: rabbit anti-Demmin polyclonal antibody (1:200; Abcam) and goat anti-rabbit IgG biotinylated antibody (Invitrogen), which was detected by streptavidin conjugated with Alexa 546 (1:250; Invitrogen), and a mouse monoclonal anti-human/mouse α-smooth muscle actin (αSMA) directly labeled with Cy3 antibody (1:250; Sigma). Basement membrane component collagen type IV was detected with rabbit anti-collagen type IV polyclonal antibody (1:200; Serotec); apoptosis was detected with rabbit anti-mouse cleaved caspase-3 (1:100; Cell Signaling Technology); sinusoidal endothelial cells were detected with rabbit anti-mouse Lyve1 (1:200; Reliatech); and goat anti-rabbit IgG conjugated with Alexa 546 (1:500; Invitrogen), as well as directly biotinylated monoclonal mouse anti-mouse Stabilin 2 (1:500). Details of morphologic and vascular analyses as well as whole liver imaging and perfusion analysis are outlined in the Supplementary Data.

Biochemical and molecular biological techniques

Protocols for standard quantitative PCR (qPCR), microarray analysis, Western blot analysis, and ELISA measurements are summarized in the Supplementary Data.

Statistical analysis

GraphPad Prism software was used. Data were analyzed by unpaired Student t test unless otherwise indicated. Values were expressed as mean ± SD. P < 0.05 was considered statistically significant.

Results

Adenoviral Cre-driven hepatocellular carcinogenesis leads to the formation of multinodular tumors

Inducible AST transgenic mice (iAST) express the SV40 TAg oncogene under the control of the albumin promoter. Yet, the oncogene is not transcribed due to the insertion of a floxed stop cassette between the promoter and the oncogene (22). Oncogenic transformation of hepatocytes is induced by excision of the floxed stop cassette, which is mediated by the hepatotropism of tail vein injected adenovirus expressing Cre recombinase (22).

Adenoviral Cre recombinase delivery induced acute hepatitis and parenchymal destruction at 1 week postinjection (Supplementary Fig. S1A). Oncogenic transformation with atypical hepatocytes appeared at 2 weeks postinjection (Fig. 1A, left) accompanied by amelioration of the acute hepatitis with persistent immune cell infiltration (Fig. 1A, right). The nuclear–cytoplasmic ratio shifted at 4 weeks postinjection with the emergence of nodular structures and fully developed HCC nodules at 9 weeks postinjection (Supplementary Fig. S1A).

Noninvasive CT imaging identified multifocal liver tumor nodules of varying sizes after 5 to 8 weeks (Fig. 1B and C). CT data were validated by histologic analyses of sectioned liver samples (Fig. 1D, left). SV40 TAg was expressed within the tumor nodules as well as in single cells, indicating an early tumorous transformation of hepatocytes (Fig. 1D, right and Supplementary Fig. S1B). Nonparenchymal cells did not express SV40 TAg. Notably, SV40 TAg-expressing lesions were positive for markers of malignant hepatocellular transformation in humans, including glypican 3 and HSP70 (Fig. 1E and F; ref. 24). Taken together, the iAST-induced tumorigenesis proved to be a versatile mouse model of hepatocarcinogenesis enabling temporal (time of virus delivery), and spatial (albumin promoter) induction of hepatocarcinogenesis in adult mice resembling the multifocal tumorigenesis observed in human HCC.

Tumor nodules establish a functional neovasculature with increasing immaturity during tumor progression

Human HCC is a highly vascularized tumor. Increased circulating levels of angiogenic factors such as VEGF and Angiopoietin-2 (Ang2) have been reported in patients with HCC and correlate with poor prognosis (25–28). Correspondingly, tumor-bearing iAST mice revealed signs of active angiogenesis: Circulating levels of VEGF were upregulated in iAST-induced tumor-bearing mice (Fig. 2A). VEGF-A expression was restricted to hepatocytes, as previously described (29), in normal livers and upregulated in nontransformed hepatocytes outside the tumorigenic area as well as in cells within larger nodules at later stages (Supplementary Fig. S2). Ang1 and even more so Ang2 mRNA and protein levels were strongly upregulated in tumorigenic livers of iAST mice (Fig. 2B and C). To quantitatively assess the tumor angiogenic response in relation to tumor progression, tumor nodules were categorized in three groups based on different nodule size, namely small (histologic surface area <0.1 mm²), intermediate (0.1–0.2 mm²), and large (>0.2 mm²). Surprisingly, no changes in total tumor vessel area

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within tumor nodules were observed during tumorigenesis. Notably, even the smallest nodules contained microvessels (Fig. 2D), suggesting that tumor angiogenesis did not occur from expansively growing avascular nodules. Instead, tumor cells in growing nodules coopted and remodeled preexisting sinusoidal vessels, similar to the recently reported cooptive growth of liver metastasizing tumors (30). This conclusion was also confirmed by histologic analyses, which revealed a grossly altered tumor microvascular architecture with enlarged, strongly CD31-positive intratumoral microvessels (Fig. 2E), compared with the low CD31 expression of liver sinusoidal endothelial cells (LSEC) as has similarly been observed in a constitutive SV40-TAg HCC model (31). These CD31-positive microvessels were well embedded in a type IV collagen-positive basement membrane in tumor nodules of different sizes (Fig. 2E and F). In addition, tumor vessels at all stages were well perfused and tumor nodules showed little to no signs of necrosis (Supplementary Fig. S3), indicating the presence of a functional vascular network at all analyzed tumor stages.

Analysis of intratumoral vessel maturation by staining for mural cell markers identified profound changes during tumorigenesis: Microvessels in smaller nodules stained prominently for the pan-pericyte marker desmin (55%–60%), whereas microvessels in larger nodules were characterized by lower pericyte coverage indices (20%–25%; Fig. 2G and H). In contrast, the fraction of microvessels positive for the pericyte activation marker αSMA was significantly larger in big nodules compared with small nodules (20% vs. 10%; Fig. 2I and J). This pattern of desmin-high/αSMA-low levels in small nodules and desmin-low/αSMA-high levels in large nodules reflected an increasing immaturity of the tumor microvasculature during tumor progression. The same marker combination has also been observed in Lewis lung carcinomas (32). Taken together, the vascular analysis of growing tumors identified a functional vascular network in early stages of tumorigenesis with signs of increasing microvascular immaturity during tumor progression.

**Sorafenib treatment inhibits tumor growth, improves vascular architecture, and enhances survival**

The multikinase inhibitor sorafenib has some efficacy as monotherapy in patients with late-stage HCC (6).
consequently examined its therapeutic efficacy in the preclinical iAST model of hepatocarcinogenesis. To this end, mice were staged by magnetic resonance imaging and treatment was initiated as soon as tumors became detectable. Daily treatment with sorafenib (100 mg/kg) dramatically reduced tumor growth compared with vehicle-treated animals (Fig. 3A–D). To assess the microvasculature in the different treatment groups, livers were perfused through the portal vein with microfil cast. Sorafenib treatment resulted in the maintenance of the typical hierarchical branching structures similar to control-treated mice (Fig. 3E).

Next, we examined the preclinical efficacy of a lower dose of sorafenib (60 mg/kg) as maintenance therapy that had previously been studied as long-term treatment (33). Early initiation of sorafenib maintenance therapy (5 weeks after tumor initiation) resulted on average in a four-fold extended OS compared with the vehicle control group (Fig. 3F). Of note, mice in the sorafenib-treated group died or were sacrificed because of poor health condition and not as a direct consequence of tumor growth. These mice displayed severe weight loss, partial necrosis and hemorrhage in the liver, and lung bleeding after 80 days of treatment; effects that can similarly be observed in

Figure 2. Expression of angiogenic factors and vascular characteristics during iAST-induced HCC progression. A, VEGFA ELISA showing increased levels of circulating VEGF in the serum of tumor-bearing mice. B, qPCR showing upregulated expression of Ang1 and Ang2 in livers with tumor nodules (n = 3–4 per group). C, Western blot analysis displaying increased protein levels of Ang1 and Ang2 in tumorigenic whole liver lysates. D, quantitative analysis of the intratumoral microvasculature, revealing no changes in tumor vascular area in different size tumor nodules (<0.1, 0.1–0.2, and >0.2 mm²; N = 5–6, n = 10–20 nodules per experimental group). E, representative images of cryosections double-stained for CD31 (green) and type IV collagen (red). F, quantitative analysis of type IV collagen and CD31 costained areas (n = 5–6, n = 20–40 nodules per experimental group). G, representative images of cryosections double-stained for CD31 (green) and desmin (red). H, quantitative analysis of desmin-covered microvessels of total intratumoral CD31-positive microvessels (n = 5–6; n = 10–20 nodules per experimental group). I, representative images of cryosections double-stained for CD31 (green) and αSMA (red). J, quantitative analysis of αSMA-covered microvessels of total intratumoral CD31-positive microvessels (n = 5–6; n = 10–20 nodules per experimental group). Scale bar, 200 μm; ELISA data were analyzed by Mann–Whitney U test (n = 4–5). *, P < 0.05; **, P < 0.01; *** P < 0.001; error bar, SD; n, tumor nodule; CV, central vein.
human patients (Supplementary Fig. S4). A delayed initiation of sorafenib treatment (upon MRI detection of tumor nodules) still resulted in a significant extension of OS, albeit with lower efficacy (Fig. 3F). The analysis of transcriptomic changes during sorafenib treatment identified the downregulation of Hbegf and Hgf expression, whereas cMet was upregulated (Fig. 3G). The data demonstrate a potent preclinical efficacy of sorafenib in the iAST model of hepatocarcinogenesis with reduced tumor growth and a largely preserved organ vascular architecture. The therapeutic response closely resembled the treatment outcome in human patients with earlier sorafenib administration, resulting in improved clinical efficacy.

The antitumorogenic effect of sorafenib results from its antiangiogenic activity

Although sorafenib has been shown to inhibit tumorigenic Raf signaling, treatment of iAST mice had only minimal effects on bRaf and cRaf as well as p44/42 MAPK (Fig. 4A–C). In contrast, Akt phosphorylation was prominently reduced upon sorafenib treatment (Fig. 4D). Moreover, expression and phosphorylation of the angiogenic receptor tyrosine kinases VEGFR2 and PDGFRß was strongly downregulated in the livers of sorafenib-treated tumor bearing iAST mice (Fig. 4E–H). This antiangiogenic effect resulted in the downregulation of Ang2 mRNA and protein and stabilized expression of the Angiopoietin receptor Tie2 despite a downregulation of Tie2 mRNA levels (Fig. 4F and H). VEGF expression was prominently upregulated upon sorafenib treatment, both, at the mRNA and protein level in serum and liver lysates (Fig. 4H–J) as well as in the tumorigenic areas in the liver (Supplementary Fig. S5) possibly due to hypoxia (Supplementary Fig. S6), suggesting a compensatory mechanism in response to treatment. Sorafenib treatment had no effect on the resting organ vasculature outside tumor nodules as evidenced by unaltered expression

Figure 3. Analysis of the effect of sorafenib on tumor growth in the iAST model. A, representative MR images of livers with detectable tumor nodules (arrowheads) at the start of sorafenib treatment. B, representative images of livers from normal nontreated mice, vehicle- and sorafenib-treated mice 14 days after treatment initiation (asterisk in A and B shows the same nodule). C, macroscopic images of livers from normal nontreated, vehicle-, and sorafenib-treated mice obtained 14 days after initiation of sorafenib. Note reduced overall tumor burden in the sorafenib-treated liver. D, quantitative analysis of tumor growth in vehicle- and sorafenib-treated mice (100 mg/kg; n = 7–8 per group). D, microcast of livers from normal nontreated, vehicle-, and sorafenib-treated mice displaying the vascular architecture of the liver vascular tree. F, Kaplan–Meier curve of vehicle-treated mice (black line), mice with early onset of sorafenib treatment (blue line; initiation of therapy 5 weeks after adenoviral Cre delivery), and late onset of treatment (red line; initiation of therapy upon MRI detection of tumor nodules, 60 mg/kg; n = 7–11 per group). E, qPCR of Hbegf, Hgf, and cMet in normal mice, as well as in vehicle-treated and sorafenib-treated tumor bearing mice (n = 5 per group). *P < 0.05; **P < 0.01; ***P < 0.001; error bar, SD; L, left; R, right; Sp, spinal cord; St, stomach; A, aorta; Gb, gall bladder.
of the sinusoidal endothelial cell markers Lyve1 (34) and Stabilin 2 (Supplementary Fig. S7; ref. 35). Together, these data show that sorafenib exerted its antitumorigenic effect in the iAST model of hepatocarcinogenesis primarily at the microvascular level as an inhibitor of tumor-associated angiogenesis.

Sorafenib treatment inhibits pericyte recruitment and leads to intratumoral microvessel regression

The antiangiogenic effects of sorafenib could be due to effects on endothelial cells, pericytes, or both. On the basis of the prominent downregulation of PDGFRβ expression upon sorafenib treatment (Fig. 4B), we performed additional qPCR and Western blot analyses to examine the effect of sorafenib on intratumoral pericytes. Expression of most pericyte markers was downregulated upon sorafenib treatment (desmin, αSMA, Ng2, CD248; Fig. 5A).

To analyze the structural features of endothelial cells and pericytes upon treatment in more detail, we assessed vessel characteristics by immunofluorescence stainings for CD31, αSMA, desmin, as well as the basement membrane component type IV collagen. Sorafenib treatment resulted in a significant reduction of intratumoral microvessel area (Fig. 5B) and a reduced number of vascular branchpoints compared with vehicle-treated animals (Fig. 5C). Whole mount image analysis revealed that the overall architecture of larger vessels was preserved in the sorafenib-treated group, whereas the vascular tree in the tumors of control mice was characterized by disorganized and chaotropic vessels (Fig. 5D). On the microvascular level, sorafenib treatment resulted in a strong reduction of αSMA (Fig. 5E–G) and desmin-covered microvessels (Fig. 5H and I). Concomitantly, significantly increased numbers of αSMA-positive and desmin-positive pericytes without microvascular association (i.e., no CD31 colocalization) were detected in tumor nodules of sorafenib-treated iAST mice (Fig. 5G and J) demonstrating that sorafenib treatment potently blocked intratumoral microvessel maturation by inhibiting pericyte recruitment. Furthermore, sorafenib treatment resulted in a decrease of type IV collagen deposition around the tumor microenvironment.
intratumoral microvessels (Fig. 5K–M). However, we identified a strong increase in the number of type IV collagen–positive empty sleeves upon sorafenib treatment, indicating active regression of sprouting neovessels, presumably of non-pericyte–covered immature capillaries. Collectively, the detailed vascular analyses identified a dual antivascular mechanism of action of sorafenib, which was characterized by an inhibition of pericyte recruitment to endothelial cells and regression of immature microvessels.

**Sorafenib leads to rapid intratumoral microvessel regression**

The pronounced antiangiogenic effects of sorafenib in the iAST model prompted us to analyze in more detail the
temporal kinetics of the observed vessel regression phenotype. No detectable microvascular changes were observed within 12 and 16 hours of sorafenib treatment (Fig. 6A). Thereafter, massive vascular regression was rapidly initiated after 24 hours of therapy induction, resulting in a vessel phenotype comparable with the 14-day end point (Fig. 6A). At this time point, desmin-positive pericytes were left within type IV collagen–positive empty sleeves (Fig. 6B). After 6 days of treatment, the dense network of intratumoral microvessels as seen in the control group was remodeled to a pruned tree of larger diameter vessels in the sorafenib-treated group (Fig. 6C and Supplementary Fig. S8). The temporal analysis of key regulators of the angiogenic cascade by qPCR in the different treatment groups revealed a significant time-dependent decrease in Vegfr2 and Tie2 expression, most likely reflecting the reduction of the absolute numbers of endothelial cells upon treatment. Vegfa expression increased gradually over time, indicating a compensatory upregulation mechanism in response to the antiangiogenic effects. Surprisingly, the Tie2 ligands Ang1 and Ang2 were most dynamically regulated being strongly downregulated as rapidly as 24 hours after initiation of therapy (Fig. 6D).

IGF1/IGF1R/IRS signaling is a potential driver of tumor progression during sorafenib treatment

We performed microarray analyses of iAST tumors from the different experimental groups to identify additional mechanisms of action in sorafenib treatment over time (Supplementary Fig. S9; original data deposited in the Gene Expression Omnibus database; accession no. GSE54857). Sorafenib treatment led to an increase of insulin-like growth factor 1 (Igf1; Fig. 7A and B) and cMet expression (Fig. 7B), whereas

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Figure 6. Rapid vascular changes upon sorafenib treatment. A–C, confocal images of CD31/cleaved caspase-3 double-stained cryosections from tumorous livers 12, 16, and 24 hours after induction of sorafenib treatment showing vascular regression with little evidence of apoptotic endothelial cell death. B, confocal images of triple-stained cryosections (CD31/desmin/type IV collagen) from tumorous livers of vehicle-treated and 24-hour sorafenib-treated mice showing a reduction of microvessel density with remaining desmin-positive pericytes in type IV collagen–positive empty sleeves (arrowheads). C, confocal images of CD31–stained cryosections from tumorous livers of vehicle-treated and 6-day sorafenib-treated mice showing stable vessels with vascular pruning and few capillary sprouts (arrowheads) following treatment. D, qPCR analysis of Vegfa, Vegfr2, Ang1, Ang2, and Tie2 at different times after vehicle and sorafenib treatment (n = 5 per group). Scale bar, 50 µm. ***, P < 0.001; error bar, SD; n, tumor nodule.
Hgf expression was decreased after 14 days as well as after long-term sorafenib treatment (Fig. 7B). Correspondingly, following a sharp decline of circulating IGF1 upon tumor induction, sorafenib treatment led to an increase of circulating IGF1 over time (Fig. 7C). Concomitantly, analysis of IGF1R receptor expression in the iAST model revealed a prominent gradual increase of IGF1R in tumor nodules during long-term sorafenib treatment (Fig. 7D). Activated insulin receptor substrate (IRS), the substrate for active IGF1R signaling, was correspondingly upregulated in the long-term sorafenib-treated samples compared with the 14 day sorafenib-treated samples as evidenced by Western blot analysis of tumorigenic liver lysates (Supplementary Fig. S10). Similarly, phospho-Akt was upregulated in the LT-sorafenib–treated samples, indicating that the tumorigenic areas in these samples regained proliferative potential. To validate the increase in IGF1 as a putative mechanism of sorafenib to overcome tumor growth reduction, lower doses of sorafenib (20 and 40 mg/kg) were given to late stage mice (upon MRI detection). While 20 mg/kg resulted on average in a survival advantage of 6 days, 40 mg/kg resulted in a more significant increase in survival (Fig. 7E). Mice receiving 40 mg/kg sorafenib were divided in two groups: One group (progressing) was analyzed at end stage, when the abdomen was increased in size and the second group (slow progressing) was analyzed before end stage (no visible swelling of the abdomen). Side effects such as severe weight loss were not observed in these...
two groups (Supplementary Fig. S11). As evidenced in Fig. 7F and G, Igf1 mRNA and protein from whole liver lysates were analyzed by qPCR and ELISA, respectively, and revealed an increase in Igf1 mRNA and protein level in the "progressing" group and more so in the "slow progressing" group. Taken together, the data provide evidence for a possible role of the IGFI/IGF1R/IRS signaling as a possible escape mechanism during continuous sorafenib treatment.

Discussion

The dramatic developments in cancer genome analysis lead to the discovery of tumor targets at an unprecedented pace. In turn, the translation of preclinical target identification and validation research into novel therapies is slow and as inefficient as never before. Preclinical mouse tumor models play a central role in the clinical translation of such targets. Yet, the limited availability of mouse models that truthfully mimic the course and progression of human tumors and their response to therapy has emerged as one of the most rate-limiting bottlenecks of translational oncology research.

There is also need for better mouse tumor models because the information flow is increasingly not going unidirectional from bench to bedside, but in circle from the bedside back to the bench when clinical observations require mechanistic analysis in defined experimental models (36). Likewise, the advancement of personalized medicine has promoted the concept of coclinal trials, which similarly depends on the availability of robust and reliable preclinical tumor models (37).

The bench-to-bedside-and-back paradigm has prototypicaly shaped the field of antiangiogenesis research. Antiangiogenic therapy was originally pioneered in preclinical tumor models to starve tumors to death by driving the intratumoral vasculature into regression (38). Yet, only the clinical translation of antiangiogenic therapy has revealed that vessel normalization by selective pruning of immature tumor vessels and subsequent facilitation of chemotherapy is a major determinant of antiangiogenic therapy in human tumors (39). Today, the clinical efficacy of antiangiogenic tumor therapy continues to be rather modest and further advancements such as the rational, mechanism-based implementation of antiangiogenic combination therapies are hampered by the limited availability of relevant tumor models.

We have in the present study characterized the angiogenic properties of the recently established Cre recombinase-inducible albumin promoter SV40 T-antigen transgenic model (iAST) of hepatocellular carcinogenesis (22) and exploited it for the study of the antiangiogenic and antitumorigenic properties of sorafenib on HCC. Tumor growth is in this model initiated by the intravenous injection of Cre recombinase-expressing adenovirus. The adenoviral transfer selectively targets hepatocytes, in which Cre recombinase excises a floxed stop cassette, leading to expression of the T-antigen under the control of the albumin promoter. Unlike constitutive T-antigen HCC models, this approach gives the model a number of unique properties that give it robustness and versatility: First, the inducible character enables a unique temporal versatility allowing tumor initiation even in aged or in pretreated mice (e.g., tumor initiation on top of CCl4-induced fibrosis). Second, titration of adenoviral dosage allows calibration of the system, leading to tumor development with 100% penetrance in a multifocal manner resembling the development of human HCC. Third, the multifocal character facilitates comparison of different tumor stages in a single tumorspecific liver. Fourth, combined with noninvasive imaging techniques, the model can conveniently be employed for therapy experiments.

Considering the intense vascularization of HCC, we focused our temporal analysis of the iAST model on vascular changes during tumor progression. As the key driver of angiogenesis in most tumor models, VEGF expression was upregulated during tumor progression. Furthermore, both, Ang1 and Ang2 were remarkably upregulated in tumorspecific livers. The intense angiogenic activity was associated with distinct processes of vessel cooption, remodeling, and vascular expansion during tumor progression with accompanying phenotype changes of the angiogenic vasculature. Previous work had shown that endothelial cell transdifferentiation is a major pathogenic event in human HCC and in the iAST model in the transition from cooptive to angiogenic tumor vascularization characterized by the loss of differentiation markers of LSEC (40). The present study identified an increasing vascular immaturity during tumor progression characterized by distinct pericyte populations with an immature gene expression signature (desmin-low/αSMA-high), which has previously been reported in other tumor models, including the RipTag model (32, 41). Interestingly, these changes occurred in the absence of major changes in intratumoral microvessel density. Moreover, unlike many other tumor models, intratumoral vessels in the iAST model were well perfused and not leaky during early nodule formation.

Sorafenib treatment of iAST tumor nodules resulted in very rapid antivasculature effects evident as early as 24 hours after initiation of therapy. Sorafenib treatment appeared to drive intratumoral endothelial cells into rapid regression, leaving behind type IV collagen–positive empty sleeves, which were still filled with pericytes. Selective VEGFR blockade in a spontaneous RipTag2 mouse model has similarly been shown to result in endothelial cell-depleted vessels sparing the pericycle (42). Yet, sorafenib targets pericytes by inhibiting PDGFβR, which has been demonstrated in vitro before (43). This led to altered expression of the pericycle markers αSMA, desmin, NG2, and CD248. These transcriptional changes could reflect drop out or reduced motility of pericytes (44). The number of pericytes decreased throughout the 14-day treatment. Yet, the overall effect was less severe compared with endothelial cells. Recently, inhibition of pericytes in addition to endothelial cells in the tumor vasculature has been shown to exert beneficial therapeutic effects (45). Further analysis is needed to determine as to what extent the remaining pericytes might contribute to the development of resistance, metastasis, or other pathways that could stimulate tumor regrowth.

Sorafenib is currently the only systemic treatment for non–resectable late-stage HCC patients. Yet, the clinical efficacy is limited and resistance eventually develops as it has been observed in preclinical models (32). It is noteworthy that
pro-oncogenic factors such as IGF1/IGF1R (46), cMet (47), and Id1 (48) were upregulated in the iAST model upon sorafenib treatment. Currently, several clinical trials inhibiting cMet and VEGFR together are underway (49) and results await the outcome regarding tumor behavior and patient survival. Importantly, IGF1 levels increased gradually during the response to treatment before end stage and before developing severe side effects. As inhibition of the IGF1/IGF1R pathway has been shown to reduce HCC growth in vivo (50), it is an important finding concerning sorafenib therapy. The iAST model may therefore be suitable to further advance combination therapies and to systematically screen for emerging resistance mechanisms during therapy.

In conclusion, we describe in the present study, the unique properties of the inducible iAST HCC tumor model as a versatile experimental system for the study of HCC progression and the response to therapy.

Note Added in Proof
A study was published in Cancer Discovery since submission of this manuscript demonstrating that VEGF-A amplified HCC are highly sensitive to sorafenib treatment (51). The findings of this study are highly compatible with the findings of our study showing that sorafenib treatment is preferentially acting anti-angiogenic with a very rapid dropout of endothelial cells as early as 24h after the initiation of treatment (Fig. 6). According to the Cancer Discovery publication, VEGF-amplified tumors promote tumor progression through an endothelial cell-derived angiocrine signaling loop, which is compatible with recent findings by our laboratory demonstrating a proliferation-regulating function of endothelial cells on hepatocytes during liver regeneration (52). Collectively, both studies strongly support a rationale for further development of combination therapies for HCC involving sorafenib and other antiangiogenic drugs—and in consideration of the observed rapid compensatory upregulation of VEGF expression following sorafenib treatment (Fig. 6 of this study).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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