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Abstract: Tumor-associated blood vessels differ from normal vessels and proteins present only on tumor vessels may serve as biomarkers or targets for anti-angiogenic therapy in cancer. Comparing the transcriptional profiles of blood vascular endothelium from human invasive bladder cancer with normal bladder tissue, we found that the endothelial cell-specific molecule endocan (ESM1) was highly elevated on tumor vessels. Endocan was associated with filopodia of angiogenic endothelial tip cells in invasive bladder cancer. Notably, endocan expression on tumor vessels correlated strongly with staging and invasiveness, predicting a shorter recurrence-free survival time in non-invasive bladder cancers. Both endocan and VEGF-A levels were higher in plasma of patients with invasive bladder cancer than healthy individuals. Mechanistic investigations in cultured blood vascular endothelial cells or transgenic mice revealed that endocan expression was stimulated by VEGF-A through the phosphorylation and activation of VEGFR-2, which was required to promote cell migration and tube formation by VEGF-A. Taken together, our findings suggest that disrupting endocan interaction with VEGFR-2 or VEGF-A could offer a novel rational strategy to inhibit tumor angiogenesis. Further, they suggest that endocan might serve as a useful biomarker to monitor disease progression and the efficacy of VEGF-A-targeting therapies in patients with bladder cancer.

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Abstract

Tumor-associated blood vessels differ from normal vessels and proteins present only on tumor vessels may serve as biomarkers or targets for anti-angiogenic therapy in cancer. Comparing the transcriptional profiles of blood vascular endothelium from human invasive bladder cancer with normal bladder tissue, we found that the endothelial cell-specific molecule endocan (ESM1) was highly elevated on tumor vessels. Endocan was associated with filopodia of angiogenic endothelial tip cells in invasive bladder cancer. Notably, endocan expression on tumor vessels correlated strongly with staging and invasiveness, predicting a shorter recurrence-free survival time in non-invasive bladder cancers. Both endocan and VEGF-A levels were higher in plasma of patients with invasive bladder cancer than healthy individuals. Mechanistic investigations in cultured blood vascular endothelial cells or transgenic mice revealed that endocan expression was stimulated by VEGF-A through the phosphorylation and activation of VEGFR-2, which was required to promote cell migration and tube formation by VEGF-A. Taken together, our findings suggest that disrupting endocan interaction with VEGFR-2 or VEGF-A could offer a novel rational strategy to inhibit tumor angiogenesis. Further, they suggest that endocan might serve as a useful biomarker to monitor disease progression and the efficacy of VEGF-A-targeting therapies in patients with bladder cancer.

Introduction

Bladder cancer is the fifth most common cancer in the developed world. It has been estimated that in 2008, 386,300 persons were diagnosed with bladder cancer worldwide and 150,200 succumbed to the disease (1). Bladder cancer has been classified into superficial (pTa, pT1 and CIS) and muscle-invasive (pT2-4) cancer based on whether tumor infiltration extends to the muscular bladder wall (2). However, based on genetic (3) and expression data (4) of bladder cancers of different stages, the revised WHO classification proposes a distinction of non-invasive (pTa) and invasive (pT1-4) bladder cancers. Non-invasive bladder cancers (pTa) have a low risk of progression but a high risk of recurrence. To date there are no clinical parameters that reliably predict tumor recurrence or progression. Despite the treatment of invasive bladder cancer with radical cystectomy (5), up to 50% of patients develop metastases and five-year survival is low (6). Increased microvessel density (7) and high expression of vascular endothelial growth factor A (VEGF-A) correlate with poor prognosis (8), progression (9, 10) and shorter survival of bladder cancer patients (11). We aimed at elucidating differences at the molecular level between tumor-associated blood vasculature and normal vasculature, that might serve as therapeutic targets or as prognostic biomarkers. Therefore, we isolated blood vascular endothelial cells (BECs) from bladder cancer and normal bladder tissue, using immuno-laser capture microdissection (i-LCM), and performed a comparative microarray expression analysis.

We found that endocan (endothelial cell-specific molecule 1) was one of the most strongly upregulated genes in tumor BECs. Endocan is a secreted proteoglycan (12) that is upregulated by growth factors and chemokines in vitro (13, 14) and on tumor

vasculature in several types of cancer (15-17), with diverse suggested biological roles (18). Using RT-qPCR and immunohistochemistry, we found that endocan was strongly upregulated on tumor vascular endothelium and that its expression correlated with the invasiveness of bladder cancer. Significantly elevated endocan levels were measured in the plasma of patients with invasive bladder cancer compared to healthy individuals. Importantly, we found that VEGF-A induces endocan expression *in vitro* and *in vivo* via VEGF receptor-2 (VEGFR-2) and that endocan knockdown in BECs inhibited VEGF-A-induced tube formation, migration and VEGFR-2 phosphorylation. Therefore, endocan appears to be an important mediator of tumor angiogenesis induced via the VEGF-A-VEGFR-2 axis and might serve as a novel biomarker in bladder cancer.

Material and methods

Tumor and plasma samples

Clinically annotated frozen and paraffin embedded tissue samples of bladder cancers and normal bladder tissue as well as plasma samples were obtained from the University Hospital Zurich. The tissue collection was approved by the University Hospital Zurich Ethics Committee (SPUK GGU- USZ Ethics Committee KEK-StV-Nr 02/09) and informed written consent was obtained from each patient. Control plasma samples were obtained from healthy blood donors. Additionally, we used a bladder cancer tissue microarray (TMA) containing 143 samples from 91 patients (67 male, 24 female; age range: 29 to 97 years) from the Institute of Pathology, University Hospital Erlangen. The TMA consisted of 89 papillary non-invasive (pTa

and papillary urothelial neoplasms of low malignant potential (PUNLMP)) and 54 papillary invasive (pT1-4) tumors. Blood vessels were identified based on their typical morphology by a board-certified pathologist (P.W.), and only vessels within tumors were analyzed to reduce heterogeneity. Endocan expression on blood vessels was semi-quantitatively assessed by grading the intensity of the staining on the majority of vessels as absent (0), weak (1+; endocan staining weaker than nuclear staining) or strong (2+; endocan staining stronger than nuclear staining). The statistical significance was computed using Fisher's exact test. The Kaplan–Meier log-rank test was used to analyze recurrence-free survival of patients with non-invasive bladder cancer (pTa; n=40) with strong endocan expression versus those with weak or absent endocan expression.

Immuno-laser capture microdissection (i-LCM) of blood vessels from bladder tissue

Frozen sections of invasive bladder cancer (5 patients; pT1-pT4) and matched normal (tumor-adjacent) bladder tissue were fixed in acetone for 1 min at 4°C. Blood vessels were stained using biotinylated rabbit anti-human von Willebrand factor (vWF) antibody (0.25 mg/ml; Dako Cytomation) and Cy3-streptavidin (1:100) in a mixture of buffers A and B (all Life Technologies) containing Protector RNase inhibitor (2U/µl; Roche). Slides were dehydrated in 75% ethanol, 95% ethanol, 100% ethanol and xylene. Immediately afterwards, i-LCM was performed using near infrared-laser based Arcturus Veritas LCM (Life Technologies). Up to 1 mm^2 of immunostained blood vessels were isolated per sample and immediately lysed in Buffer RLT Plus (Qiagen) containing 3% β-mercaptoethanol.

RNA isolation, cDNA generation and microarray hybridization of i-LCM samples

RNA was isolated using the RNeasy Plus Micro Kit (Qiagen), amplified and converted to cDNA using the whole transcriptome amplification kit Ovation Pico WTA System (Nugen). Then, cDNA was purified using the QIAquick PCR purification kit. Biotin-labeled cDNA targets were hybridized to Human Exon ST 1.0 arrays (Affymetrix) and arrays were scanned according to the manufacturer's protocol. The microarray data is available at GEO under accession number GSE41614.

Immunohistochemical and immunofluorescent stainings

Paraffin-embedded tissue sections and the TMA were dewaxed in xylene and rehydrated using graded percentages of ethanol. After incubation in 3% H_2O_2 for 10 min, sections were boiled in Tris-EDTA buffer, pH=9 for 20 min. Staining for endocan and VEGF-A was performed using mouse anti-human endocan MEP08 antibody (10 µg/ml, Lunginnov) and rabbit anti-human VEGF-A A-20 antibody (4 µg/ml, Santa Cruz). Biotin-labeled horse anti-mouse (5 µg/ml) and biotin labeled goat anti-rabbit (15 µg/ml) antibodies were used as secondary antibodies. Immunoreactive signals were amplified by formation of avidin–biotin peroxidase complexes and visualized using 3-amino-9-ethylcarbazole (AEC) or 3,3-diaminobenzidine (DAB). Nuclear counterstaining was performed with hematoxylin. A rabbit anti-human vWF antibody (15.5 µg/ml) and Alexa Fluor 594-labeled donkey anti-rabbit antibody (1:200) were used for immunofluorescence stainings, followed by Hoechst (10 µg/ml) nuclear staining.

Confocal imaging

Immunofluorescent double staining was performed on 80-µm thick sections according to (19), using the endocan antibody MEP08 and the vWF antibody, followed by Alexa Fluor 594-labeled donkey anti-mouse (1:500) and Alexa Fluor 488-labeled donkey anti-rabbit (1:500) antibodies. Images were acquired with a Leica SP2 confocal microscope using a 63×1.4 NA Plan Apochromat objective with oil immersion lens. 3D-reconstruction was performed using the Imaris software (Bitplane Scientific Software). The resulting image represents a stack of 47 sections (Z step of 0.244 µm) with a total physical length of 11.23 µm. The voxel height was 0.173 µm with a zoom of 2.693.

ELISA assays

Plasma samples from 60 healthy donors (median 58 y, range: 36.8-75 y, male 87%, female 13%) and 53 patients with invasive bladder cancer (median 70.8 y; range: 45.3-84 y; male 79%, female 21%) were analyzed by ELISA for endocan (Diyek endomark H1; Lunginnov) and VEGF-A (Platinum; eBiosciences), using a Sunrise Tecan microplate reader (Tecan). For BEC lysates, the protein concentration of samples was adjusted, using a bicinchoninic acid assay (Thermo Scientific).

VEGF-A transgenic mice and VEGFR-2 blocking experiments

K14/VEGF-A transgenic (Tg) mice that express mouse VEGF-A164 under control of the K14 promoter (20, 21) were bred and housed in the animal facility of ETH Zurich. Untreated FVB wild-type mice were used as controls. Experiments were performed in accordance with animal protocol 149/2008 approved by the Kantonales Veterinaeramt Zurich. Mouse ears were harvested from 24-weeks old VEGF-A Tg mice (n=6) and

wild-type (WT) mice (n=6) and homogenized in RLT buffer containing 1% β-mercaptoethanol. VEGF-A Tg mice (15-weeks old) were also treated with 800 μg of rat anti-mouse VEGFR-2 blocking antibody DC101 (ImClone) or control rat IgG (Sigma) i.p. every other day for 7 days.

Isolation of dermal BECs by fluorescence-activated cell sorting (FACS)

Dermal BECs were isolated from the ears of VEGF-A Tg mice treated with DC101 (n=5) or control IgG (n=3), and of wild-type FVB mice (n=6) using high-speed cell sorting as described (22).

Cell culture

Primary human dermal BECs (23) and human umbilical vein endothelial cells (HUVECs; Promocell) were seeded onto fibronectin-coated culture dishes (10 μg/ml; BD Biosciences) and were cultured in endothelial cell basal medium (EBM; Lonza) supplemented with 20% FBS (Invitrogen), 2 mM L-glutamine (Fluka), 10 μg/ml hydrocortisone (Fluka), and antibiotic/antimycotic solution (Invitrogen). The BEC media also contained endothelial cell growth supplement (Promocell). Cells of passage 6-8 were used.

Cell treatment with proangiogenic factors and VEGFR-2 blocking experiments

Twelve hours before stimulation, media were replaced with EBM containing 1% FBS. Cells were then treated with recombinant human VEGF-A (20 ng/ml) or FGF-2 (20 ng/ml; R&D Systems) or both. Cells treated with vehicle were used as controls. For some experiments, cells were pre-incubated with human monoclonal antibody IMC-1121B against VEGFR-2 (20 μg/ml; Imclone) or with control human IgG for 4h,

followed by incubation with VEGF-A (20 ng/ml) for 24h. Then, cells were washed and lysed in RLT buffer containing 3% β-mercaptoethanol.

RNA isolation and cDNA generation from endothelial cell lysates and mouse ears

RNA was isolated from mouse ear homogenates and from BECs using the RNeasy Mini kit and treated with RNase-free DNase (all from Qiagen). cDNA was generated from 1 μg RNA using the High-Capacity cDNA Reverse Transcription kit (Life Technologies).

Quantitative real-time PCR

The expression of mouse and human endocan was quantified by TaqMan real-time PCR with the AB 7900 HT Fast Real- Time PCR System and the $2^{-\Delta\Delta C}{}_t$ method (24). Taqman probe/primer sets for mouse (Mm00469953_m1) and human endocan (Hs00199831_m1) and for mouse CD34 (Mm00519283_m1) were pre-designed by Life Technologies. Each reaction was multiplexed with β-actin (4326315E) or B2M (4326319E) for human endothelial cells, or β-actin (4352341E, all from Life Technologies) for mouse samples. Expression of endocan and CD34 was normalized to the expression of the reference gene.

siRNA knockdown of endocan

siRNA electroporation of HUVEC was performed using the Amaxa Basic Nucleofactor Kit for primary endothelial cells (Lonza). The knockdown efficiency of three different siRNA constructs against endocan was evaluated (ID: 136192, ID: 19124, ID: 19216). Knockdown using siRNA ID: 136192 showed the strongest reduction of endocan mRNA (data not shown). Therefore, siRNA ID: 136192 and the

no. 1 siRNA control nontargeted siRNA (NT siRNA; all Ambion) were used. After 24 or 36h, supernatants were collected and cells were lysed in RLT buffer containing 3% β-mercaptoethanol, or in a hypotonic PBS solution containing 1 mM MgCl2, 1 mM CaCl2, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail (Roche) as described (25).

Western Blot analyses

Supernatants were concentrated using a Centricon Ultracel YM-10 membrane filter (Millipore). Proteins were precipitated as described (26). Supernatant samples containing 100 μg of protein and cell lysate samples containing 10 μg of protein (assessed using the BCA protein assay) were subjected to SDS-PAGE using 10% acrylamide separating gels, 1.0 mm. Proteins were transferred to Immobilon-P transfer membranes (Millipore) and stained using a biotinylated goat anti-human endocan antibody (0.2 μg/mL; R&D Systems) or rabbit anti-human (p1175)VEGFR-2 (D55B11; 1□1000), VEGFR-2 (55B11; 1:1000) or ERK1/2 (137F5; 1□1000; all Cell Signaling) antibodies and a streptavidin-bound horseradish peroxidase conjugate (1:5000) or horseradish peroxidase-coupled anti-rabbit antibody (1:5000). Stained proteins were detected using the ECL Plus Western Blotting Detection System (GE Healthcare). Equal loading was confirmed by Ponceau S (Sigma) staining and by staining for vWF (6 μg /mL; (27)) or ERK 1/2.

Tube formation and migration assays

The capillary tube formation assay was performed as described (28) with minor modifications. Matrigel (BD Biosciences) was added to 96-well plates (40 μl per well) and let solidify for 30 min at 37°C. HUVECs were electroporated with endocan

siRNA or NT siRNA (100 µl of cell suspension containing 15,000 cells/well), seeded on top of Matrigel in sextaplicates and incubated for 12h. Images were acquired with a digital camera (AxioCam MRm, Carl Zeiss) mounted on an inverted microscope (Axiovert 200M, Carl Zeiss). The total length of tube-like structures per well was measured using ImageJ. Endothelial cell migration was assessed using a Scratch monolayer wound-healing assay (29). HUVECs were electroporated using endocan siRNA or NT siRNA and seeded into fibronectin-coated wells in quadruplicates (120,000 cells/well). At confluency, two cross-shaped scratches were made in each well using a sterile 200 µl pipette tip. Cells were washed and VEGF-A (20 ng/ml) or plain medium were added. Images of crosses were taken immediately and 25 h later. The surface areas of the cell-free zones were measured and the % scratch closure was determined using TScratch software (29).

Statistical analyses

Statistical analyses were performed using Prism version 5.00 (GraphPad Software, Inc.). For most comparisons, a two-tailed unpaired Student's t-test was performed. The differences in plasma concentrations of VEGF-A and endocan were analyzed with the Mann-Whitney U test. Spearman rank correlations were made to test for the association between endocan and VEGF-A concentrations in plasma. The AUC, sensitivity, specificity, positive predictive value, negative predictive value and likelihood ratio were calculated from the receiver operating characteristics (ROC) curve. The comparison between endocan expression in non-invasive and invasive cancers was calculated using Fisher's exact test. Survival data were plotted using the Kaplan-Meier method and analyzed using the log-rank (Mantel-Cox) test. Differences were considered statistically significant at $P<0.05$.

Results

Endocan expression is upregulated in invasive bladder cancer-associated blood vascular endothelial cells

We isolated endothelial cells from bladder cancer-associated vessels and from vessels in normal surrounding bladder tissue by i-LCM. Taqman-based quantitative real-time PCR showed that endocan mRNA expression was 1,000- to 100,000-fold higher in tumor-associated BECs than in normal BECs (Fig. 1A). Immunohistochemical staining revealed that the endocan protein was present on tumor-associated blood vessels, but not on the blood vessels of normal bladder tissue (Fig. 1B). Confocal imaging of immunofluorescently stained sections of invasive bladder cancer revealed that endocan was associated with the surface of the plasma membrane of BECs. Staining was particularly intense at the filopodia of endothelial tip cells, which typically are vWF-negative ((30); Fig. 1C).

Endocan expression is higher on blood vessels of invasive than noninvasive bladder cancer and is associated with reduced recurrence-free survival

No staining for endocan was detected in normal bladder tissue (Fig. 2A). We next analyzed a TMA consisting of non-invasive (n=89) and invasive bladder cancers (n=54). Of these, 116 samples contained a sufficient number of vessels for analysis of endocan expression which was semi-quantitatively assessed by grading the intensity of the staining as absent (0) (Fig. 2B), weak (1+) (Fig. 2C) or strong (2+) (Fig. 2D). We observed that endocan expression was heterogeneous in individual punches (Fig. 2E). Vessels outside the tumors were negative (Fig. 2F), therefore only vessels within tumors were analyzed to reduce heterogeneity (Fig. 2G). 53 out of 70 samples of non-

invasive bladder cancer had no or weak staining for endocan whereas 17 samples had strong staining. From 46 invasive samples, 23 had no or weak staining and 23 had strong staining. The difference in frequencies between these groups was significant (P=0.005) (Fig. 2H), indicating that endocan expression on tumor blood vessels increases with invasiveness of bladder cancer. Kaplan-Meier analysis of 40 samples of pTa cancers showed that patients with strong endocan expression (n=12, N_{events}=9) had a reduced recurrence-free survival compared to those with no or weak endocan expression (n=28, N_{events}=14; P=0.079) (Fig. 2I).

Endocan is elevated in plasma of patients with invasive bladder cancer

We next tested whether the increased endocan expression in invasive bladder cancers would also be apparent in plasma. The mean concentration of endocan was significantly higher (P<0.001, Mann-Whitney U test) in the plasma of patients with invasive bladder cancer (0.79 ng/ml; range 0.23-3.53 ng/ml; n=53) than of healthy volunteers (0.43 ng/ml; range 0-1 ng/ml; n=60) (Fig. 3). Using ROC (receiver operating characteristic) curve and AUC (area under curve), we determined the ability of endocan levels to allow for distinction between patients with invasive bladder cancer and healthy subjects. The AUC, sensitivity and specificity were 0.76, 64% and 80%, respectively, with a cut off of 0.63 ng/mL. The positive predictive value (PPV) and the negative predictive value (NPV) were 74% and 72%, respectively, with a likelihood ratio of 3.208.

VEGF-A is increased in tumor tissue and plasma of patients with invasive bladder cancer

It has been reported that *in vitro,* endocan expression is induced by VEGF-A (13, 16). We therefore asked whether the enhanced endocan expression on tumor blood vessels might be due to increased VEGF-A production. Immunostaining of invasive bladder cancers revealed VEGF-A expression by tumor cells (Fig. 4A, right panel, white arrows). VEGF-A was also present on tumor-associated blood vessels, likely reflecting VEGF-A bound to its receptors (31) (Fig. 4A, right panel, black arrows). In contrast, VEGF-A was not detected in normal bladder tissue (Fig. 4A, left panel) and in non-invasive bladder cancer (Fig. 4A, middle panel). VEGF-A levels in plasma of healthy blood donors were significantly lower (mean: 0.12 ng/ml, range: 0.01-0.4 ng/ml, n=60) than in patients with invasive bladder cancer (mean: 0.28 ng/ml, range: 0.04-0.89 ng/ml, n=53; $P<0.001$, Mann-Whitney U test; Fig. 4B). The AUC, sensitivity and specificity were 0.83, 68% and 90%, respectively, with a cut off of 0.1895 ng/mL. The positive predictive value and the negative predictive value were 86% and 76%. The likelihood ratio was 6.72. Combining the 113 samples analyzed, we found a significant correlation of VEGF and endocan levels ($P=0.004$; Fig. 4C).

Endocan expression is upregulated by VEGF-A *in vitro* and *in vivo*

After treatment of human BECs with VEGF-A (20 ng/ml) for 24h, endocan expression was significantly increased at the mRNA (1.58-1.80-fold; Fig. 5A) and protein level in cell lysates (mean 10.17, range: 9.48-10.62 ng/ml; control treated: mean 7.79 ng/ml, range: 6.21-8.73 ng/ml; Fig. 5B). Increased endocan levels were also detected in culture supernatants (mean 41.2 ng/ml, range: 36.45-44.1 ng/ml; control treated: mean 33.8 ng/ml, range: 32.52-34.59 ng/ml; Fig. 5C). FGF-2 alone or in combination with VEGF-A did not alter endocan mRNA expression. Pre-treatment of BECs with a VEGFR-2 blocking antibody inhibited induction of endocan by

VEGF-A at the mRNA (0.74-0.76-fold compared to control IgG; Fig. 5D) and protein level (mean 30.6 ng/ml in supernatants, range: 29.07-32.55 ng/ml; control IgG treated: mean 39.42 ng/ml, range 37.74-40.8 ng/ml; Fig. 5E).

To investigate whether VEGF-A might also enhance endocan expression by BECs *in vivo*, we analyzed skin samples obtained from homozygous VEGF-A Tg mice which have elevated VEGF-A levels in the skin (21). Endocan mRNA expression was strongly increased (27.2-67.6-fold; Fig. 5F) in the skin of transgenic mice (n=6) compared to wild-type mice (n=6). To investigate whether this was solely the consequence of increased blood vessel numbers, we specifically isolated blood vessel-derived endothelial cells from the ear skin by FACS. We found upregulation of endocan in endothelial cells derived from VEGF-A Tg mice (2.91-5.34-fold, Fig. 5G), while expression of the vascular marker CD34 was unchanged (Fig. 5H). Treatment of VEGF-A Tg mice with the VEGFR-2 receptor blocking antibody DC101 resulted in reduced endocan expression in *ex vivo* isolated endothelial cells compared to the control IgG group (0.2-0.46-fold, Fig. 5I); CD34 expression was not affected (Fig. 5J).

Endocan knockdown in BECs inhibits VEGF-A-induced tube formation, migration and VEGFR-2 phosphorylation

We next investigated whether and how endocan might contribute to VEGF-A-induced angiogenesis. The biological functions of proteoglycans often depend on the interactions of their glycosaminoglycan chains with protein ligands such as cytokines and growth factors (32, 33). Therefore, we investigated whether silencing of endocan would affect VEGF-A effects on endothelial tube formation or migration. Electroporation of HUVECs with endocan siRNA strongly reduced endocan

expression at the mRNA (Fig. 6A) and protein levels (Fig. 6B). The total length of tube-like structures formed by HUVECs on growth factor-rich Matrigel was significantly reduced upon endocan knockdown compared to control siRNA (-45%; P=0.012; Fig. 6C). In a monolayer wound healing ("scratch") assay, migration of HUVECs was not affected by endocan knock-down (data not shown). However, when cells were stimulated with VEGF-A, silencing of endocan abolished the migration-inducing effect of VEGF-A (reduction of wound closure by 53% compared to control siRNA knockdown, P=0.007) to levels observed in non-VEGF-treated controls (Fig. 6D, E). Importantly, knockdown of endocan, followed by incubation of HUVECs with VEGF-A for 10 min, resulted in a strong reduction of VEGFR-2 phosphorylation, whereas the total amount of VEGFR-2 was unchanged (Fig. 6F). These findings indicate that endocan is necessary for VEGF-A-induced signaling and VEGFR-2 phosphorylation.

Discussion

Here, we established a novel method for efficient isolation of tumor-associated blood vessels from invasive bladder cancers and matched normal bladder tissue using i-LCM. Transcriptional profiling revealed endocan as a gene whose expression was strongly increased on tumor-associated blood vessels, in line with gene expression profiling studies in breast (34), lung (35), and thyroid (36) cancers.

Endocan mRNA and protein expression was strongly increased on blood vessels of invasive bladder cancers compared to normal bladder tissue and non-invasive bladder cancers. Importantly, non-invasive bladder cancer patients with strong endocan

expression had a shorter recurrence-free survival than those with absent or weak endocan expression. Currently, the standard follow-up for non-invasive bladder cancer includes cystoscopy combined with cytological examination at intervals of 3 to 6 months, depending on tumor malignancy and previous recurrence rate. Cystoscopic examinations are unpleasant, time-consuming, expensive, and may have serious side effects such as infections and damage to the urethra (37). Cytology is characterized by a high specificity but a low sensitivity. So far, there are only few promising molecular markers that might predict recurrence-free survival (38). Our study suggests that endocan might be an additional marker that could allow some prediction how often patients need to undergo cystoscopic examination.

The increased endocan concentrations in the plasma of patients with invasive bladder cancers indicate that endocan might serve as an additional biomarker to evaluate the overall prognosis of invasive bladder cancers. However, additional studies are needed to investigate whether there are consistent differences of plasma endocan levels between invasive and non-invasive cases.

As endocan is a soluble proteoglycan, we asked where it may be localized within the tumor-associated blood vessels. Using high-resolution confocal imaging, we found that endocan was associated with cell membranes of vascular endothelial cells. Particularly high amounts of endocan were associated with filopodia of endothelial cells. Filopodia are typically present on tip cells and are needed for their guidance and motility during angiogenesis (39). In agreement with our findings, expression of endocan was previously also found on tip cells in developing mouse retinas (40, 41).

Tip cells are responsive to VEGF-A and other angiogenic factors and guidance molecules (42), and tumor-associated vessels form extensive filopodia (19). Thus, we asked whether endocan may play a role in migration and sprouting of endothelial

cells. Indeed, when endothelial cells were stimulated with VEGF-A, siRNA-mediated endocan silencing abolished the migration induced by VEGF-A. In contrast to a previous report (43), we found that siRNA-mediated endocan silencing also abolished endothelial tube formation. This discrepancy is likely due to different assay conditions used (HUVECs embedded between two layers of collagen gel versus plating on top of a solidified Matrigel in our study). We also found that expression of endocan by BECs was upregulated after VEGF-A treatment *in vitro*, whereas incubation with FGF-2 had no effect on endocan mRNA or protein production. The different responsiveness of HUVECs (17) and BECs (our study) to FGF-2 likely reflects functional differences between large vessel-derived endothelial cells (HUVEC) and microvasculature-derived endothelial cells (BECs).

Endocan carries a dermatan sulfate chain attached to its serine 137 (18). Dermatan sulfate is a linear polysaccharide which consists of repeating disaccharide units composed of sulfated N-acetylgalactosamine and either glucuronic or iduronic acid (44). Both the sulfates and the carboxylates of the uronic acids are negatively charged at physiological pH, thus providing binding sites for signaling molecules comprising positively charged amino acids such as cytokines and growth factors. Interactions between signaling molecules and glycosaminoglycan chains are often highly specific and may serve to create high local concentrations of signaling molecules, to prolong their half-life by protecting them from proteolytic degradation and to facilitate their binding to their cognate receptors (45). It has been shown that dermatan sulfate chains of endocan bind hepatocyte growth factor (HGF) (46) and promote its mitogenic activity on HEK293 cells (47). However, we previously found that HGF only had a weak effect on the proliferation of BECs (48). In contrast, our novel finding that

siRNA-mediated silencing of endocan resulted in strongly reduced phosphorylation of VEGFR-2 - the major transmitter of VEGF-A's effects on endothelial cells - upon incubation with VEGF-A, indicates a major role of endocan in mediating the effects of VEGF-A. Endocan is secreted by endothelial cells upon VEGF-A stimulation and binds VEGF-A on the cell surface, facilitating its interaction with VEGFR-2 and increasing the intensity of the VEGF-A signal. It remains to be investigated whether endocan might also have paracrine effects on non-vascular cells (16).

Taken together, these results indicate that VEGF-A induces endocan expression in endothelial cells, which in turn enhances VEGF-A-induced endothelial cell migration and angiogenesis. The interrelation of endocan expression with the presence of VEGF-A on one side and the angiogenesis-inducing function of VEGF-A, supported by endocan, on the other side could make endocan an ideal biomarker for monitoring the therapeutic response to treatment with VEGF-A-targeting anti-angiogenic agents. This concept is further supported by the strongly reduced endocan levels after systemic treatment of VEGF-A Tg mice with an anti-VEGFR-2 antibody.

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Figure Legends

Figure 1. **Endocan expression is upregulated in invasive bladder cancer-associated blood vascular endothelial cells.** (A) BECs were isolated from frozen sections of invasive tumors (tBEC) and adjacent normal (nBEC) tissue from five patients. Endocan mRNA was quantified by qRT-PCR and normalized to the mean expression in nBEC. Data represent mean ± SD. ***P<0.001. (B) Paraffin sections of invasive bladder cancer and normal bladder tissue were stained immunohistochemically for endocan (red) and immunofluorescently for vWF as a blood vessel marker, and with hematoxylin or Hoechst (nuclear counterstains). Scale bars: 100 μm. (C) Z-stack slice of a confocal image of invasive bladder cancer immunofluorescently stained for endocan (red), vWF (green) and Hoechst (blue). 3D reconstruction of confocal z-stack using Imaris software. Scale bars: 10 μm.

Figure 2. **Endocan expression is higher on blood vessels of invasive than noninvasive bladder cancer and is associated with reduced recurrence-free survival.** Endocan expression on blood vessels (arrows) was semi-quantitatively assessed by grading the staining intensity. Representative images of normal urothelium (A) and of bladder cancers with absent (B), weak (C) and strong (D) endocan expression (brown) are shown. Representative images of a sample TMA core (E), weak endocan expression in the stroma (F) and strong expression in the tumor (G). Scale bars: 100 μm. (H) Quantification of endocan expression levels in non-invasive (pTa) and invasive (pT1-pT3) bladder cancers of a TMA of 116 bladder cancers. **P < 0.01. (I) Kaplan-Meier recurrence-free survival curves of patients with

non-invasive bladder cancer (pTa; n=40) with strong endocan expression (2+) versus those with weak or absent endocan expression (0, 1+).

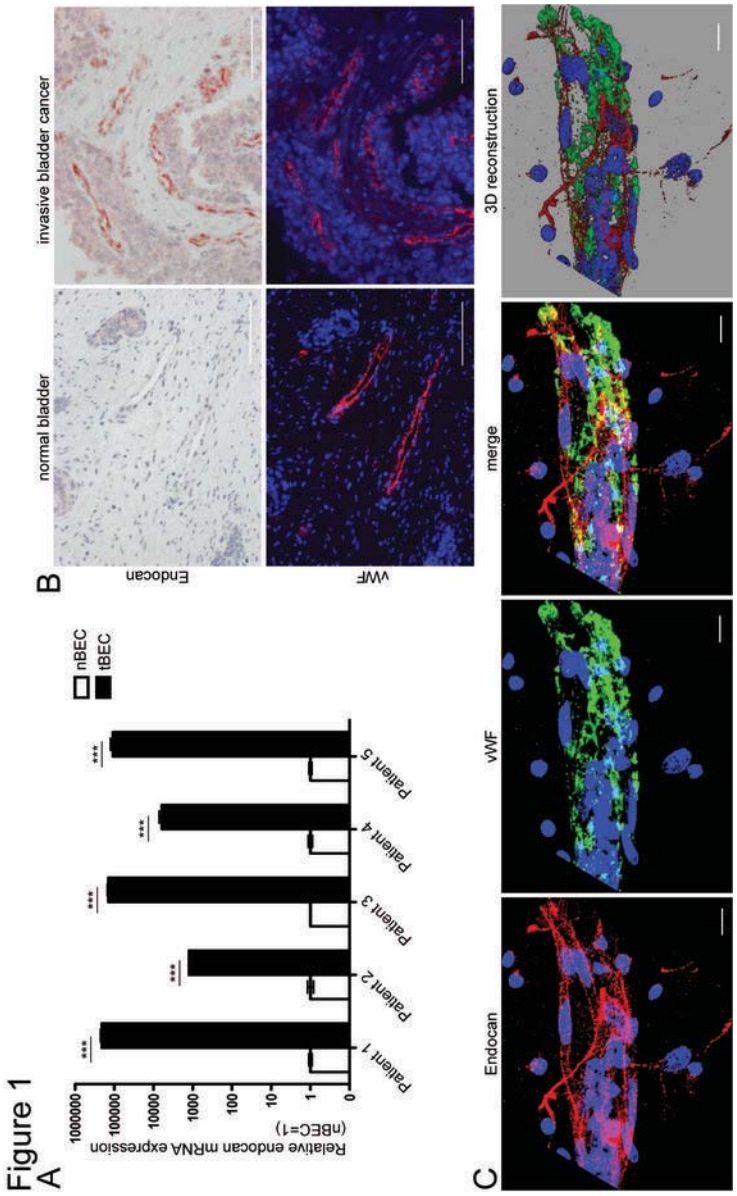
Figure 3. **Endocan is elevated in plasma of patients with invasive bladder cancer.** Endocan was measured in plasma of patients with invasive bladder cancer (n=53) and in healthy volunteers of similar age (n=60) by ELISA. Dots represent patient samples. Horizontal lines represent mean values. ***P<0.001.

Figure 4. **VEGF-A is increased in tumor tissue and plasma of patients with invasive bladder cancer.** (A) Paraffin sections of normal bladder tissue (left), non-invasive (middle) and invasive bladder cancer (right) were stained immunohistochemically for VEGF-A (top panels; brown), immunofluorescently for vWF (bottom panels; red) and with hematoxylin or Hoechst (nuclear counterstains). Higher magnification of boxed area shows VEGF-A expressed by tumor cells (white arrows) and VEGF-A associated with tumor endothelium (black arrows). Scale bars: 100 μm. (B) VEGF-A was measured in plasma of patients with invasive bladder cancer (n=60) and healthy volunteers of similar age (n=53) by ELISA. Dots represent patient samples. Horizontal lines represent mean values. ***P<0.001. (C) Significant correlation of endocan and VEGF-A levels (n=113).

Figure 5. **Endocan expression is upregulated by VEGF-A *in vitro* and *in vivo*.** Expression of endocan in primary human BECs after incubation with VEGF-A (20 ng/mL) and/or FGF-2 (20 ng/mL) for 24 h was quantified by qRT-PCR (A) and ELISA in cell lysates (B) and supernatants (C). Cells treated with medium alone were used as controls. Reduced expression of endocan mRNA (D) and protein in

supernatants (E) after treatment with a VEGFR-2 blocking antibody. Increased endocan mRNA expression in the skin (F) and in FACS-isolated BECs from skin (G) of homozygous K14-VEGF-A transgenic mice (VEGF-A Tg, n=6) compared to wild-type mice (WT, n=6). CD34 expression levels were comparable in BECs isolated from both genotypes (H). Reduced endocan expression in FACS-isolated BECs from skin of VEGF-A Tg mice (n=5) after treatment with a blocking VEGFR-2 antibody (I), compared to control IgG (n=3). BEC CD34 expression levels were comparable in both treatment groups (J). Data represent mean±SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 6. **Endocan knockdown in BECs inhibits VEGF-A-induced tube formation, migration and VEGFR-2 phosphorylation.** HUVECs were electroporated with siRNA against endocan or non-targeting siRNA (NT siRNA). Endocan knockdown was confirmed by RT-qPCR (A) and Western blot analysis (B). vWF and Ponceau staining served as loading controls. (C) Capillary tube formation in VEGF-A containing matrigel, quantitified as total tube length per well in μm, was inhibited by endocan siRNA. (D) VEGF-A induction of HUVEC migration was prevented by endocan knockdown, as assessed in a scratch wound healing assay. Scale bars: 100 μm. (E) Quantification of scratch closure (n=8 per group) using TScratch software. Data from one representative experiment are presented as mean±SD. *P<0.05, **P<0.01, ***P<0.001. (F) Endocan knockdown potently inhibited VEGFR-2 phosphorylation. HUVECs were incubated with or without VEGF-A for 10 min. Cell lysates were subjected to Western blot analysis, using anti-phospho-VEGFR-2, anti-VEGFR-2, anti-endocan and anti-ERK 1/2 antibodies. Staining for ERK1/2 served as a loading control.



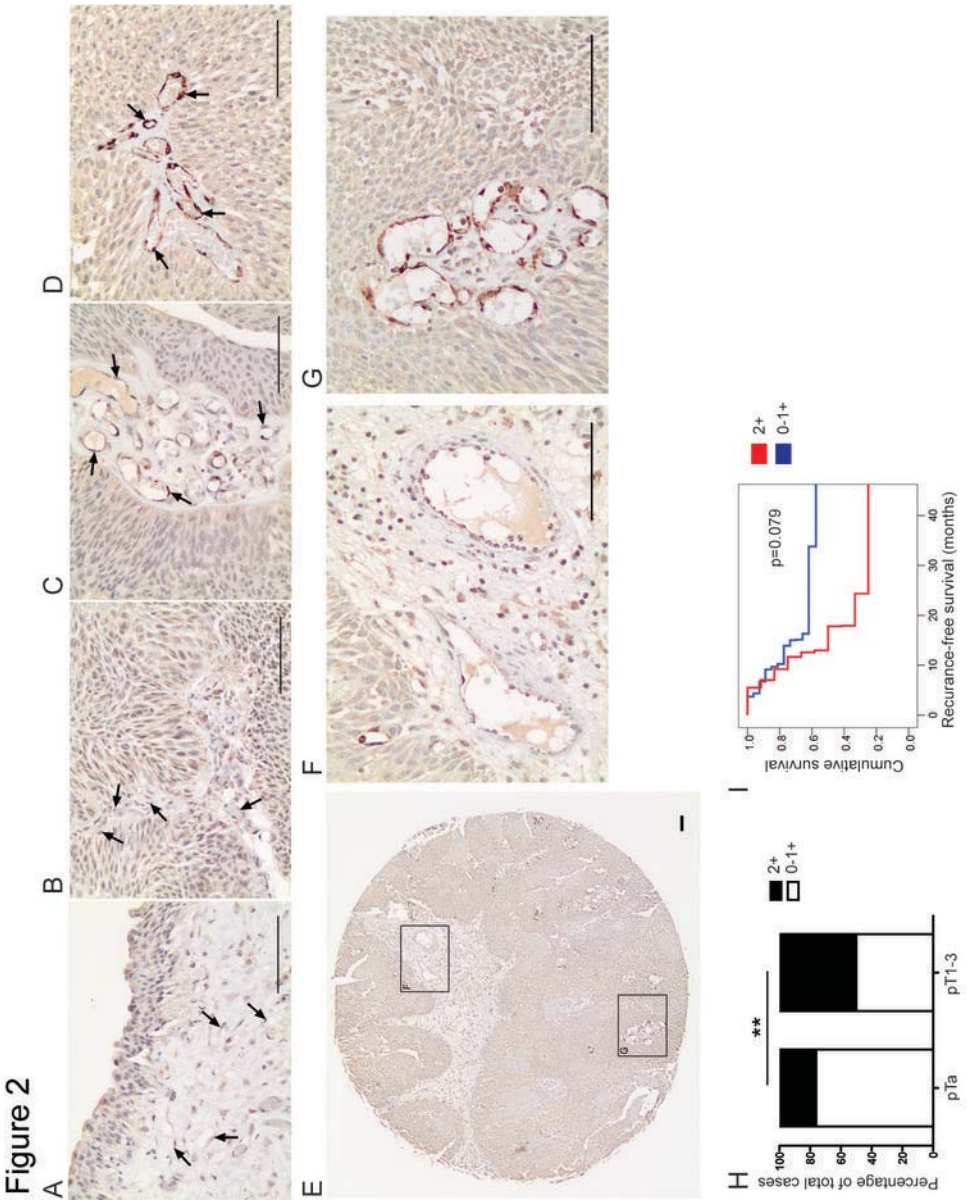


Figure 3

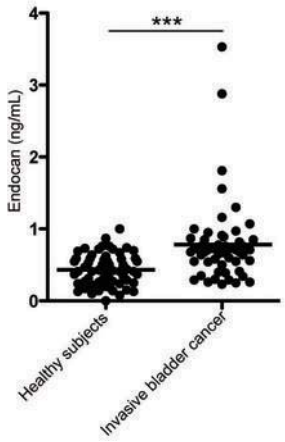


Figure 4