Endosialin-Expressing Pericytes Promote Metastatic Dissemination

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Abstract

Metastasis is a multistep process that is critically dependent on the interaction of metastasizing tumor cells with the local microenvironment. Within this tumor stroma, vessel-associated pericytes and myofibroblasts share a number of traits, including the upregulated expression of the transmembrane receptor endosialin (CD248). Comparative experiments in wild-type and endosialin-deficient mice revealed that stromal endosialin does not affect primary tumor growth but strongly promotes spontaneous metastasis. Mechanistically, endosialin-expressing pericytes in the primary tumor facilitate distant site metastasis by promoting tumor cell intravasation in a cell contact–dependent manner, resulting in elevated numbers of circulating tumor cells. Corresponding to these preclinical experiments, in independent cohorts of primary human breast cancers, upregulated endosialin expression significantly correlates with increased metastasis and poorer patient survival. Together, the data demonstrate a critical role for endosialin-expressing primary tumor pericytes in mediating metastatic dissemination and identify endosialin as a promising therapeutic target in breast cancer. Cancer Res; 76(18): 5313–25. ©2016 AACR.

Introduction

The vast majority of cancer-related mortality is due to distant site metastasis and not to primary tumor growth. Metastasis thereby marks the transition from a local to a systemic disease. The multistep nature of metastatic tumor cell dissemination and colonization is widely appreciated (1). Yet, the molecular and mechanistic understanding of individual steps of the metastatic cascade and the identification of bottlenecks that could serve as therapeutic targets is still in its infancy.

Metastatic progression is critically dependent on the interaction of metastasizing tumor cells with the cells of their microenvironment, both at the primary tumor site as well as at the site of metastasis (2, 3). Among the cells of the tumor microenvironment, stromal myofibroblasts, macrophages, and other inflammatory cells, as well as cellular constituents of blood and lymphatic vessels, i.e., endothelial cells, pericytes and vascular smooth muscle cells, comprise the tumor stroma (4, 5). Interestingly, tumor-associated pericytes and stromal myofibroblasts share a number of functional and molecular traits including the expression of the cell surface receptor endosialin (CD248).

Endosialin (CD248) is a transmembrane glycoprotein (6) that was originally described as a cell surface marker of the tumor endothelium (7, 8). However, high-resolution morphologic analyses of a range of human cancers have unequivocally demonstrated that endosialin is not expressed by endothelial cells, but by pericytes and myofibroblasts (9–13). Importantly, resting mesenchymal cells in the healthy adult have low or undetectable levels of endosialin expression. Expression is essentially restricted to activated cells of the mesenchymal lineage during embryogenesis (10, 14) as well as during pathologic states including tumor progression and metastasis (13, 15, 16), making endosialin an oncofetal protein with potential as a biomarker and a therapeutic target (17–20). In fact, clinical trials in solid tumors and lymphomas with an endosialin-targeting antibody are ongoing (www.clinicaltrials.gov/ct2/results?term=endosialin&Search=Search; ref. 21).

Functionally, endosialin regulates mesenchymal cell proliferation through PDGFRβ signaling (22, 23), which has been associated with the promigratory mesenchymal cell phenotype (24–26). Endosialin-deficient mice are, unless pathologically challenged, phenotypically normal (27), although they display some delay of developmental vascular remodeling caused by perturbed pericyte function (28, 29). Earlier studies have reported no overt difference in the growth of subcutaneous tumors in wild-type and endosialin-deficient mice (27, 28, 30). Yet, transplantable colorectal tumors grown orthotopically in endosialin-deficient mice displayed a prominent reduction in primary tumor growth, invasion, and metastasis compared

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with endosialin wild-type tumor-bearing mice (27). This striking difference between subcutaneous and orthotopic tumor growth in endosialin-deficient mice prototypically highlights the important contribution of the microenvironment to tumor progression and metastasis and identifies endosialin as a functionally relevant receptor in the tumor stroma. At the same time, pericytes have been shown to limit metastasis in the Riptag2 model of pancreatic β-cell tumorigenesis (31). Conceptually linking these two totally independent lines of research, we hypothesized that the upregulated expression of endosialin by tumor-associated pericytes may be an important contributor to tumor progression and metastasis. We consequently set out to systematically dissect the role of endosialin in the control of individual steps of the metastatic cascade. These experiments yielded unexpected mechanistic insights into the role of endosialin in regulating metastasis and identified the control of tumor cell invasation by activated pericytes as a critical and rate-limiting step.

Materials and Methods

In vivo studies

In vivo experiments were carried out under a UK Home Office Project license 70/7413 or under ethical guidelines of the local Animal Use and Care Committees approved by the Regierungspälsidium in Karlsruhe, Germany [35-9185.81/G-195/10]. Animals were housed in barriers at the animal facility of the DKFZ or in Optimice cages at the ICR. All animals were monitored on a daily basis for signs of ill health and had free access to food and water. 129/Sv mice with a global knockout of endosialin were kindly provided by Dr. David Huso (Johns Hopkins Medical Institutions, Baltimore, MD; ref. 27). Mice were backcrossed for generation 2 or more. Mice were subjected to analyses. Mice were sacrificed when they showed signs of ill health.

For spontaneous metastasis assays, 4T1 tumor cells were injected into the mammary fat pad or subcutaneously in 6–10 weeks old female WT or ENKO BALB/c or Swiss nude mice (Charles River), or C57BL/6 (DKFZ) mice. Genotypes were confirmed by PCR.

For spontaneous metastasis assays, 4T1 tumor cells were injected into the mammary fat pad or subcutaneously in 6–10 weeks old female WT or ENKO BALB/c or Swiss nude mice. LLC cells were implanted subcutaneously in the flank of 8–10 weeks old WT or ENKO C57Bl/6 mice. Tumor size was monitored by VISI imaging. Tumor volume was measured as 0.5 × length × width².

For 4T1 or LLC tumor resection, primary tumors were removed under general anesthesia. Mice with tumors not effectively removed or with subsequent tumor recurrence were removed from analyses. Mice were sacrificed at the indicated time points or when they showed signs of ill health.

Protocols for tissue processing, vascular leakage, and hypoxia assessment are described in the Supplementary Data.

Ex vivo culture of circulating tumor cells

Arterial blood was isolated by cardiac puncture and 100 µL plated in DMEM plus 10% FCS per 10-cm tissue culture plate. Tumor cell colonies were stained 14 days later with crystal violet. Plates were scanned at 300 dpi on an Epson V700 scanner and analyzed by Adobe Photoshop CS6.

Cells and antibodies

4T1 cells (ATCC, 2013). 4T1 cells expressing luciferase (4T1-Luc; provided by Dr. K. Srivastava, DKFZ, 2014). 4T1 or 4T1-Luc cells expressing GFP (4T1-GFP, 4T1-Luc-GFP) were cultured in DMEM or RPMI (Invitrogen) plus 10% FCS (Invitrogen). Lewis lung carcinoma cells (LLC, obtained from ATCC, 2006) were cultured in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin. Human MDA-MB-231-L2 expressing luciferase and GFP (MDA-MB231-Luc-GFP; provided by Dr. C. Lowry, DKFZ, 2014) cells were cultured in DMEM plus 10% FCS, 1% penicillin/streptomycin. Cell authentication by Multiplexion GmbH resulted in 98% identity towards MDA-MB-231 cells (December 2015).

Human umbilical vein endothelial cells (HUVEC, November 2015, tested by Promocell via flow cytometry and PCR) were cultured in Endopan3 medium with supplements (PAN, Biotech GmbH). The immortalized mouse endothelial cell line sEND (provided by R. Bicknell, University of Birmingham, Birmingham, United Kingdom) was cultured in DMEM plus 10% FCS. Human brain vascular pericytes (BP, November 2015, tested by ScienCell via immunofluorescence and PCR) and 10T1/2 cells (ATCC, 1997) were cultured in pericyte medium (ScienCell) plus 2% FCS, 1% of the corresponding pericyte growth supplement (PGS), and 1% penicillin/streptomycin, according to the manufacturer's protocol. BPs were infected with pGIPZ lentiviral vectors (Dharmacon) containing control (shNT, GIPZ shRNA empty vector; RHS4349) or endosialin-targeting (shEN: V2LHS_34217) shRNAs at a multiplicity of infection (MOI) of 10. 10T1/2 cells were infected with MISSION shRNA lentivirus particles (Sigma) encoding nontargeting (SHC002V) or endosialin-targeting (shEN; TRC0000098115) shRNAs at an MOI of 5. Cells were selected in puromycin. Primary mouse brain pericytes were isolated as described previously (32). All cell lines were routinely tested for mycoplasma by PCR and tested negative.

A list of antibodies and their use is provided in Supplementary Table S1.

Biochemical and molecular biological techniques

Protocols for standard quantitative PCR (qPCR), microarray analysis (original data deposited in the Gene Expression Omnibus database; accession no. GSE48008), and Western blot analysis are summarized in the Supplementary Data.

Transwell migration assay

10T1/2 cells (7.5 × 10⁴) or mouse brain pericytes (7.5 × 10⁴) on their own or with SEND endothelial cells (5 × 10⁴) were plated in pericyte medium onto the top chamber of 24-mm 8.0-µm pore Matrigel-coated Transwell filters (Corning) for 24 hours. Twenty-four hours later, 4T1-RFP cells (5 × 10⁴) were added to the top chamber in 2% FCS supplemented AdvDMEM, with 20% FCS-supplemented AdvDMEM in the bottom chamber. Twenty-four hours later, Transwells were washed, transmigrated cells detached in trypsin/EDTA, and 4T1-RFP cell number quantified on LSRII FACs analyzer.

HUVECs (1 × 10⁵) were seeded onto the top chamber of 6.5-mm/8.0-µm pore Matrigel-coated Transwells (Corning) for 24 hours. Twenty-four hours later, 4T1-RFP cells (5 × 10⁴) were added to the top chamber in 2% FCS supplemented GlutaMAX with 10% FCS-supplemented GlutaMAX in the bottom chamber. Six hours later, Transwells were washed, fixed with Roti-Histoform 4% for 10 minutes and cell number analyzed by fluorescent microscopy (×10 magnification, 6 fields per filter) and quantified by...
Endosialin does not affect metastatic seeding or colonization

On the basis of the prominent metastasis phenotype in the absence of an overt primary tumor phenotype, we hypothesized that stromal endosialin might affect metastatic seeding and/or colonization. Consequently, we studied metastatic colonization upon tail vein injection of 4T1 (Fig. 3A–D) or LLC (Fig. 3E and F) cells in WT and ENKO mice. There was no difference in lung tumor burden between experimental groups in either tumor model, indicating that stromal endosialin plays no role in metastatic seeding or colonization.

Primary tumors in ENKO mice show no defects in stromal architecture

The exclusion of an effect of endosialin on metastatic seeding and colonization prompted us to analyze in greater detail stromal attributes of primary tumors grown in WT and ENKO mice. In particular, given the well-documented upregulation of endosialin expression on pericytes in the tumor vasculature (9–13), we examined the architecture and functionality of tumor vessels. 4T1 primary tumors grown in BALB/c (Fig. 4A and B) and ENKO mice (Fig. 4C and D) revealed no significant differences in the number of tumor microvessels, total vessel area, vessel caliber distribution, or deposition of collagen IV in the vascular basement membrane between WT and ENKO mice. Likewise, vessel maturation, as assessed by pericyte coverage through αSMA and desmin staining, did not differ in tumors grown in WT or ENKO mice (Fig. 4E and F). Similarly, functional experiments showed no difference in vessel perfusion (data not shown) or permeability as monitored by leakage of Hoechst dye (Fig. 4G and H).}

Patient samples

The German 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 specimen and corresponding clinical information were exclusively provided in a pseudonymized form according to the Standard Operating Procedures of the NCT, approved by the ethic committee of the University of Heidelberg (Ethics Votes #206/207, year 2005). The UK sample collection was approved by the research ethics committee of the Royal Marsden NHS Foundation Trust.

Statistical analysis

Statistics were performed using GraphPad Prism 6. Unless otherwise stated, all numerical data are expressed as mean ± SEM. All comparisons between two groups were made using the two-tailed, unpaired Student t test, if not indicated otherwise. Unless otherwise stated in the figure legend, *, P < 0.05; **, P < 0.01 and ***, P < 0.001.

Results

Stromal expression of endosialin does not affect primary tumor growth but promotes spontaneous metastasis

To investigate the role of stromal endosialin on tumor metastasis, we compared growth and metastatic progression of orthotopic syngeneic 4T1 mammary tumors in BALB/c wild-type (WT) and endosialin-deficient (ENKO) mice. Confocal microscopy and gene expression profiling of 4T1 tumors revealed that endosialin expression on the tumor vasculature was restricted to pericytes with no detectable expression on endothelial cells (Supplementary Fig. S1A and S1B; original data deposited in the Gene Expression Omnibus database; accession no. GSE84008). No significant differences in primary tumor growth were detected (Fig. 1A), nor in primary tumor weight at necropsy (Supplementary Fig. S2A). In contrast, there was a significant reduction in spontaneous metastasis in ENKO compared with WT mice, as monitored by tumor burden in the lungs (Fig. 1B and C). Metastatic progression was limited in these experiments by the growth of the primary tumor. To circumvent this limitation and better mimic the clinical setting, 4T1 primary tumors were surgically resected at small size (day 9; ~100 mm3) and metastatic progression was traced until mice were culled for ethical reasons or the experiment terminated. Consistent with the data in Fig. 1A, there was no significant difference in the weight of the primary tumors at resection (Supplementary Fig. S2B). However, only 30.7% of WT mice, compared with 70.0% of ENKO mice, survived to termination of the experiment (day 93, Fig. 1D). Examination of lung metastatic burden revealed a significantly reduced number of pulmonary tumor nodules in ENKO compared with WT mice (Fig. 1E). Of note, surviving mice at the end of the experiment had no detectable metastatic disease in the lungs.

To exclude possible adaptive immunity-related effects in the observed metastasis phenotype, we next performed 4T1 metastasis experiments in Swiss nude WT and ENKO immunocompromised mice. Again, there was no significant difference in primary tumor growth (Fig. 1F and G and Supplementary Fig. S2C). However, metastatic burden as monitored by ex vivo IVIS imaging was significantly reduced in ENKO mice (Fig. 1H). Moreover, IVIS imaging of individual organs revealed a significant reduction in metastasis not just to the lungs, but also to liver, lymph nodes, spleen, brain, and bone (Fig. 1H and I and Supplementary Fig. S2D–S2I), indicating that the decrease in metastasis in BALB/c ENKO mice was not dependent on an intact immune system.

To validate the 4T1 metastasis phenotype in a second tumor model, Lewis lung carcinoma (LLC) cells were grown subcutaneously in C57BL/6 WT and ENKO mice and tumors were surgically resected at day 16 (~300 mm3). As with the 4T1 tumors, endosialin expression in the LLC tumor vasculature was restricted to pericytes (Supplementary Fig. S1C). There was no significant difference in primary tumor growth (Fig. 2A), but there was a significant reduction in the number of ENKO mice with histologically detectable metastases in the lungs upon termination of the experiment (day 35; Fig. 2B and C).
Figure 1.
Stromal endosialin promotes spontaneous metastasis, while not affecting primary 4T1 tumor growth. **A–E**, 4T1 cells (2 × 10⁵) were injected orthotopically into BALB/c WT and ENKO mice. **A**, primary tumor growth (n = 10–11 per group). **B**, quantification of spontaneous lung metastases from three sections per mouse. Each data point represents one mouse. °, P < 0.05. **C**, representative H&E staining of lung sections. Arrowheads, metastatic lung lesions. Scale bar, 1 mm. **D**, Kaplan–Meier survival curves after surgical removal of the primary tumor at day 9 or 10 (n = 10–13 mice per group). **E**, quantification of lung metastasis. °, P < 0.05. **F–I**, 4T1-Luc cells (1 × 10⁶) were orthotopically injected into Swiss nude WT (n = 7) or ENKO (n = 4) mice. **F**, primary tumor growth (see Supplementary Fig. S2C for primary tumor growth of second experiment; WT, n = 5; ENKO, n = 4). **G**, representative in vivo IVIS images at day 7. **H**, quantification of spontaneous metastasis to organs monitored via ex vivo IVIS imaging 14 days after surgical primary tumor removal. Each data point represents one organ (combination of two independent experiments; WT, n = 12; ENKO, n = 8 mice); see Supplementary Fig. S2D–S2I for individual organs; **∗∗∗,** P = 0.0005; Mann–Whitney test. **I**, representative ex vivo IVIS images.
proliferation, or apoptosis did not differ between WT and ENKO mice in either tumor model (Fig. 4I–L and Supplementary Fig. S4C–S4G). Finally, given that endosialin is also expressed by myofibroblasts, we assessed stromal collagen deposition in the primary tumors by Sirius red staining and the distribution and abundance of αSMA-positive myofibroblasts, which again showed no differences between the experimental groups (Supplementary Fig. S4H–S4J). Taken together, these studies revealed no discernible structural stromal differences between tumors in WT and ENKO mice that could account for the prominent metastatic phenotype of endosialin-deficient mice.

Stromal endosialin promotes tumor cell intravasation

A key role of pericytes is in stabilizing the normal vasculature (33). Given the striking upregulation of endosialin expression on tumor-associated pericytes (9–13), we next addressed whether tumor cells displayed an altered propensity to intravasate into the vasculature in WT and ENKO mice that could account for the prominent metastatic phenotype of endosialin-deficient mice.

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To test this hypothesis directly, we performed in vitro tumor cell transmigration assays (Supplementary Fig. S5A–S5F). First, 10T1/2 pericyte-like cells expressing nontargeting (shNT) or endosialin-targeting (shEN) shRNAs (Fig. 6A and Supplementary Fig. S6A) were cultured on Matrigel-coated Transwell filters for 24 hours prior to addition of 4T1-RFP tumor cells, and transmigrated cells were quantified 24 hours later by FACS analysis (Supplementary Fig. S5A). The presence of endosialin-expressing shNT 10T1/2 cells, but not shEN 10T1/2 cells with downregulated endosialin expression, enhanced transmigration of 4T1-RFP cells (Fig. 6B). To ensure that these effects were not restricted to 10T1/2 cells, the experiment was repeated using pericytes isolated from the brains of non-tumor bearing WT and ENKO mice (Fig. 6C and D and Supplementary Fig. S6B). Again, the presence of WT pericytes, but not ENKO pericytes, enhanced the transmigration of cocultured 4T1-RFP cells (Fig. 6D). To validate the tumor cell transmigration enhancing effect of pericycle-expressed endosialin, we examined the ability of human MDA-MB-231 breast cancer
cells to transmigrate across a layer of shNT or shEN human brain pericytes (Supplementary Figs. S5B and S6C–S6E). As with the mouse cell experiments, the presence of shNT-human pericytes, but not shEN-pericytes, promoted transmigration of the cocultured human tumor cells (Fig. 6E).

Next, to closer model tumor cell intravasation, we mimicked the 3D assembly of the normal vessel wall by allowing tumor cells to transmigrate across sandwich-cultured HUVECs or microvascular endothelial cells and pericytes (Supplementary Fig. S5C and S5D). As in tumors (see Supplementary Fig. S1) neither HUVEC (9) nor microvascular endothelial cells (Supplementary Fig. S7A) expressed endosialin. The presence of endosialin-expressing pericytes significantly promoted tumor cell transmigration across the endothelial cell monolayer (Fig. 6F). Equivalent results were obtained using mouse microvascular endothelial cells and shEN or shNT 10T1/2 cells (Supplementary Fig. S7B). Notably, there was no difference between tumor cell transmigration across the endothelial monolayer in the presence of pericytes with downregulated endosialin expression and across an endothelial cell monolayer without pericytes, indicating that endosialin-expressing pericytes actively promote tumor cell intravasation into the vasculature.

Figure 3.
Metastatic seeding or colonization is not affected by stromal endosialin. A–D, 4T1 cells (5 × 10^5) were injected intravenously into BALB/c WT and ENKO mice (n = 9 per group). A, quantification of tumor burden in lungs from IVIS images (day 14). Each data point represents one mouse. B, representative IVIS images. C, quantification of lung metastatic area. Each data point represents one mouse. D, representative H&E–stained lung sections. Arrowheads, metastatic lesions. Scale bar, 1 mm.

E and F, LLC cells (1 × 10^5) were injected intravenously into C57BL/6 WT (n = 11) and ENKO (n = 6) mice. E, quantification of lung colonization at day 14. Scoring: 0, no tumor; 1, small metastatic foci; 2, large metastatic foci covering 5% to 25% of section; 3, large metastatic foci covering >25% of section. Each data point represents one mouse.
F, representative images of resected lungs and corresponding H&E sections. Scale bar, 0.5 mm. ns, nonsignificant.
Vessel architecture and function in the primary tumor is not affected by stromal endosialin. A–C, 4T1 cells (2 × 10^5) were injected into BALB/c WT and ENKO mice (n = 7 per group). A, endomucin (red)- and αSMA (green)-stained tumor sections. Scale bar, 100 μm. B, quantification of blood vessel density from three random fields per section. Each data point represents one mouse. C, pericyte coverage quantified as percentage of blood vessels associated with αSMA+ pericytes from three random fields per section. Each data point represents one mouse. D–F, 4T1-Luc primary tumors in Swiss nude WT and ENKO mice (n = 5–6 per group from Fig. 1F). D, CD31 (green)- and desmin (red)-stained tumor sections. Scale bar, 100 μm. E, quantification of CD31+ vessel area per total tumor area. F, quantification of desmin+ area per total tumor area. G, quantification of Hoechst dye in 4T1-tumor-bearing WT and ENKO BALB/c mice normalized to blood vessel density (n = 4 per group). Three random fields per tumor section were analyzed. H, representative images. Scale bar, 100 μm. I, 4T1 tumor-bearing WT (n = 11) or ENKO (n = 8) BALB/c mice were injected with pimonidazole. Hypoxia was quantified as percentage of pimonidazole-positive tumor area per total tumor area. Each data point represents one mouse. J–L, 4T1-Luc primary tumors in Swiss nude WT and ENKO mice (n = 4–7 per group from Fig. 1F) were sectioned. J, quantification of necrotic tumor area per total tumor area. K, quantification of proliferation by Ki67+ cells per high power field of view. Data represents mean values from five random fields per tumor section. L, representative images of Ki67 staining. Scale bar, 50 μm. ns, nonsignificant.
Stromal cell endosialin effects on tumor cell intravasation are cell contact-dependent

To shed further insights into the mechanism by which stromal endosialin promotes tumor cell intravasation, we performed transmigration experiments in the presence of pericyte-conditioned medium (Supplementary Fig. S5E). The promotion of tumor cell transmigration by endosialin-expressing pericytes could not be recapitulated by replacing pericytes with pericyte-conditioned medium (Fig. 6G), indicating that enhanced tumor cell migration was not solely dependent on promigratory factors secreted by endosialin-positive pericytes. Consequently, we set out to determine, whether endosialin-mediated direct cell–cell interactions between pericytes and tumor cells. To this end, we performed adhesion assays with both shNT and shEN 10T1/2 pericyte-like cells and brain pericytes isolated from WT and ENKO mice. With both cells types, adhesion of 4T1-RFP cells was significantly impaired by downregulation or genetic deletion of endosialin (Fig. 6H and I).

Finally, we investigated whether a pericyte-remodeled matrix could substitute for the promigratory cell–cell contact-dependent phenotype observed with intact pericytes. 10T1/2 cells and isolated brain pericytes were allowed to remodel the matrix of Matrigel-coated Transwell filters, prior to decellularization by ammonium hydroxide treatment (Supplementary Fig. S5F). Notably, the enhanced transmigratory phenotype of 4T1-RFP cells cocultured with live shNT pericytes was not recapitulated by a pericyte-remodeled matrix (Supplementary Fig. S7C). Similarly, the increased adhesion of 4T1 cells to WT pericytes was not recapitulated in an adhesion assay where WT pericytes were replaced by WT pericyte-derived extracellular matrix (Supplementary Fig. S7D). These data indicate that the presence of live endosialin-expressing pericytes is required to induce the promigratory, cell–cell contact-dependent intravasation phenotype of tumor cells.

Endosialin expression on primary human carcinoma samples primes for metastatic spread

On the basis of the prominent role of stromal endosialin in mediating metastasis in preclinical mouse models, we assessed the clinical relevance of these experimental findings. First,
comparison of microdissected breast tumor stroma and normal adjacent stroma displayed a significantly higher level of endosialin (CD248) mRNA in the tumor stroma (Fig. 7A). Next, we stained human primary invasive breast cancer samples with two independent anti-endosialin mAbs (B1/35 and FB5; ref. 9). As illustrated in Fig. 7B, variable expression of endosialin was detected on tumor pericytes and stromal myofibroblasts. However, with the exception of a rare subset of metaplastic breast cancers, no endosialin expression was detected on tumor cells. When comparing endosialin protein and CD248 mRNA levels in the primary tumor with the metastatic stage, a significant correlation was observed between endosialin protein (Fig. 7C and Supplementary Fig. S8A) and mRNA levels (Fig. 7D) with increased incidence of metastasis. In particular, higher CD248 expression in the primary tumor was found in patients with distant metastasis to the lung, liver, and bone as compared with patients without detectable metastatic disease (Supplementary Fig. S8B). Importantly, examination of CD248 expression in microdissected breast tumor stroma (34) revealed a significant association between high stromal CD248 expression and decreased recurrence-free survival (Fig. 7E), whereas high CD248 expression in a dataset of 334 lymph-node positive breast cancers (35) was significantly associated with decreased distant metastasis-free survival (Fig. 7F). Taken together, these data indicate that endosialin expression in the primary tumor serves as poor prognostic factor for the development of metastatic disease.

Discussion

Endosialin (CD248) is a mesenchymal cell surface receptor that is widely expressed during embryonic development and downregulated in healthy adult tissues (10, 11, 14, 25). Endosialin may be abundantly expressed in the adult upon pathologic challenge, most notably by pericytes and myofibroblasts in the tumor-associated stroma (7, 9, 11, 12, 14) as well as in fibrotic (26) and atherosclerotic tissues (H.G. Augustin, unpublished data). Although endosialin was originally named TEM1 (tumor endothelial marker 1; refs. 6, 8), this was later shown to be a misleading nomenclature when different laboratories reported endosialin expression being restricted to stromal pericytes and myofibroblasts and absent from endothelial cells of various human and mouse tumors (9, 11, 12, 25, 36). The downregulated expression in the adult in combination with the striking upregulation in mouse tumors (9, 11, 12, 25, 36) was significantly associated with decreased distant metastasis-free survival (Fig. 7F). Together, these data indicate that endosialin expression in the primary tumor serves as a poor prognostic factor for the development of metastatic disease.

Activated Pericytes Promote Metastatic Dissemination

As illustrated in Fig. 7B, variable expression of endosialin was detected on tumor pericytes and stromal myofibroblasts. However, with the exception of a rare subset of metaplastic breast cancers, no endosialin expression was detected on tumor cells. When comparing endosialin protein and CD248 mRNA levels in the primary tumor with the metastatic stage, a significant correlation was observed between endosialin protein (Fig. 7C and Supplementary Fig. S8A) and mRNA levels (Fig. 7D) with increased incidence of metastasis. In particular, higher CD248 expression in the primary tumor was found in patients with distant metastasis to the lung, liver, and bone as compared with patients without detectable metastatic disease (Supplementary Fig. S8B). Importantly, examination of CD248 expression in microdissected breast tumor stroma (34) revealed a significant association between high stromal CD248 expression and decreased recurrence-free survival (Fig. 7E), whereas high CD248 expression in a dataset of 334 lymph-node positive breast cancers (35) was significantly associated with decreased distant metastasis-free survival (Fig. 7F). Taken together, these data indicate that endosialin expression in the primary tumor serves as a poor prognostic factor for the development of metastatic disease.

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As illustrated in Fig. 7B, variable expression of endosialin was detected on tumor pericytes and stromal myofibroblasts. However, with the exception of a rare subset of metaplastic breast cancers, no endosialin expression was detected on tumor cells. When comparing endosialin protein and CD248 mRNA levels in the primary tumor with the metastatic stage, a significant correlation was observed between endosialin protein (Fig. 7C and Supplementary Fig. S8A) and mRNA levels (Fig. 7D) with increased incidence of metastasis. In particular, higher CD248 expression in the primary tumor was found in patients with distant metastasis to the lung, liver, and bone as compared with patients without detectable metastatic disease (Supplementary Fig. S8B). Importantly, examination of CD248 expression in microdissected breast tumor stroma (34) revealed a significant association between high stromal CD248 expression and decreased recurrence-free survival (Fig. 7E), whereas high CD248 expression in a dataset of 334 lymph-node positive breast cancers (35) was significantly associated with decreased distant metastasis-free survival (Fig. 7F). Taken together, these data indicate that endosialin expression in the primary tumor serves as a poor prognostic factor for the development of metastatic disease.

Discussion

Endosialin (CD248) is a mesenchymal cell surface receptor that is widely expressed during embryonic development and downregulated in healthy adult tissues (10, 11, 14, 25). Endosialin may be abundantly expressed in the adult upon pathologic challenge, most notably by pericytes and myofibroblasts in the tumor-associated stroma (7, 9, 11, 12, 14) as well as in fibrotic (26) and atherosclerotic tissues (H.G. Augustin, unpublished data). Although endosialin was originally named TEM1 (tumor endothelial marker 1; refs. 6, 8), this was later shown to be a misleading nomenclature when different laboratories reported endosialin expression being restricted to stromal pericytes and myofibroblasts and absent from endothelial cells of various human and mouse tumors (9, 11, 12, 25, 36). The downregulated expression in the adult in combination with the striking upregulation in tumors makes endosialin an oncofetal protein that is, in principle, strictly based on its unique pathology-associated expression pattern, an attractive therapeutic target. Indeed, we demonstrate here, in three different preclinical models that stromal endosialin expression facilitates spontaneous distant site metastasis. To mechanistically unravel the role of stromal endosialin during individual steps of tumor progression and metastasis, we set out comparative tumor experiments in wild-type (WT) and endosialin-deficient (ENKO) mouse models and in 3D in vitro reconstitution assays. Complementing these studies with the analysis of human breast cancer expression datasets, we show that (i) enhanced spontaneous metastasis in WT mice requires upregulated endosialin expression on tumor-associated pericytes to promote tumor cell intravasation into the circulation, (ii) endosialin mediates direct cell–cell interactions of tumor-associated pericytes and tumor cells to promote tumor cells transmigration across an endothelial monolayer, and (iii) breast cancer patients with high primary tumor endosialin expression have significantly higher rates of metastasis and reduced recurrence-free survival.

Previous studies addressing the role of pericytes in tumorigenesis have focused on ablating pericytes (37, 38) or blockade of PDGFβR signaling in pericytes by genetic targeting (31), pharmacologic inhibition (39), PDGFβR–blocking antibodies (37, 40) or PDGF-B-binding oligonucleotide aptamers (41). All these strategies resulted in loss of pericyte coverage on the tumor vasculature, subsequent vascular destabilization and, in most cases, a reduction in tumor volume. Intriguingly, where it was examined, this vascular destabilization and inhibition of tumor growth, potentially enhancing tumor hypoxia and inducing tumor cell EMT, was accompanied by increased metastatic spread (31, 37, 38). Indeed, low pericyte coverage in clinical samples is associated with poor patient prognosis (42, 43). These findings are reminiscent of recent studies demonstrating that two independent genetic strategies to reduce myofibroblasts in the stroma of pancreatic cancers resulted in enhanced tumor aggressiveness (44, 45). However, while genetic deletion of Shh resulted in enhanced tumor growth (45), pharmacologic targeting of the Hh pathway improved drug delivery and reduced tumor invasion and metastasis (46, 47). These contrasting data reinforce the notion that stromal effects on tumor progression and metastasis may be highly contextual and that stromal cells can play both tumor-promoting and tumor-suppressing roles dependent on the tumor type and location. This has led to the concept that it may be more effective to deploy strategies to "normalize" or de-differentiate tumor-associated stromal cells rather than target them for destruction.

Consistent with this concept, unlike targeting PDGFβR or ablating pericytes, treatment of syngeneic tumor bearing human CD248 knock-in mice with the anti-human endosialin antibody MORAb-004 did not result in a reduction in vessel number or destabilization of the vasculature, as monitored by endothelial cell viability, pericyte coverage, and integrity of the basement membrane, but significantly impaired the growth of B16-F10 tumors following subcutaneous or intravenous inoculation (36). Importantly, in the study reported here, the impairment in metastatic dissemination in the ENKO mice was not associated with gross alterations in the tumor vasculature. Primary tumors in both syngeneic and immunocompromised WT and ENKO mice were indistinguishable in terms of vessel architecture including, as previously observed in the developing retinal vasculature of ENKO mice (28), no detectable loss in pericyte coverage. Similarly, there were no significant differences in vascular patency in WT and ENKO tumors as monitored by vessel perfusion, vascular leakage, and levels of primary tumor hypoxia and necrosis, indicating that an inability to upregulate endosialin expression in the tumor stroma has minimal impact on vascular integrity. Importantly, in primary tumors from ENKO mice, the number or distribution of αSMA-expressing myofibroblasts was not altered, suggesting that the expression of endosialin by myofibroblasts is not the driving force of tumor cell dissemination and intravasation. Consistent with this lack of stromal disruption, no differences in primary tumor growth in WT and ENKO mice were detected in the three tumor models used in this study. Previous studies also reported no differences in LLC or B16-F0 subcutaneous primary tumor growth in WT and ENKO mice (27, 28) or following intracranial inoculation of C57MG glioblastoma cells (30). In contrast, when HCT116 colorectal tumor fragments were orthotopically grafted onto the intestinal surface, tumors in the ENKO mice were reduced in volume and showed reduced local invasion and metastasis.
Figure 6.
Endosialin-expressing pericytes promote tumor cell intravasation in a cell contact-dependent manner. See Supplementary Fig. S5 for experimental setup. 

A, shNT and shEN 10T1/2 cells stained for endosialin (green) or phalloidin (red). Scale bar, 75 μm. 

B, quantification of transmigrated 4T1-RFP cells through a Transwell layer of shNT and shEN 10T1/2 cells after 24 hours. Control wells contained no 10T1/2 cells. 

C, brain pericytes isolated from WT or ENKO BALB/c mice stained for endosialin (red) or endomucin (green). Scale bar, 75 μm. (Continued on the following page.)
Endosialin expression in primary human breast cancer stroma primes for metastatic spread. A, comparison of endosialin (CD248) expression in six matched cases of microdissected breast tumor stroma and normal adjacent stroma (34). ***, P = 0.0003, two-tailed paired t test. B, human breast cancer samples stained for endosialin using BI/35 (0.6-mm invasive breast cancer cores) or FB5 (whole invasive ductal carcinoma samples) antibody. Images illustrate negative, weak, or strong endosialin expression in tumor stroma. Arrowheads, endosialin-positive pericytes and myofibroblasts. Scale bar, 100 μm. C, quantification of stromal endosialin protein in invasive ductal carcinoma samples correlated to the lymph node metastasis status at the time of primary tumor removal and the distant metastasis status approximately 10 years after primary tumor removal. Forty-seven patient samples were analyzed. Eight patients, no metastasis; 19 patients, low metastatic potential (pN1a, 1–3 lymph nodes show tumors of 0.2 cm size; 6 patients developed distant metastasis after primary tumor and lymph node removal); 12 patients, high metastatic potential (pN1b, >1a, 4–10 lymph nodes showed tumors of 0.2 cm size; 5 patients developed distant metastasis after primary tumor and lymph node removal); 19 patients, distant metastasis (M1) to lung, brain, liver, and bone. * P < 0.05; ** P < 0.01. Two-tailed paired t test. D, qPCR quantification (n = 45) showing relative CD248 mRNA levels in primary tumor samples from patients with no metastasis (n = 9), low metastatic potential (n = 9, pN1a), high metastatic potential (n = 8, pN1b), and distant metastasis (n = 17, M1). * P < 0.05. Two-tailed paired t test. E, Kaplan–Meier curves of recurrence-free survival assessed by the Finak and colleagues (34) dataset. P = 0.0474, log-rank Mantel–Cox. F, Kaplan–Meier curves of distant metastasis-free survival assessed using publicly available data from Györffy and colleagues (35) showing high metastatic potential (pN1b, >1a, 4–10 lymph nodes showed tumors of 0.2 cm size; 5 patients developed distant metastasis after primary tumor and lymph node removal); 19 patients, distant metastasis (M1) to lung, brain, liver, and bone. * P < 0.05; ** P < 0.01. Two-tailed paired t test. G, quantification of MDA-MB-231-LM2 transmigration as described in H except that instead of BP, conditioned medium from shNT or shEN BP was added to the top well 5 hours prior to addition of MDA-MB-231-LM2 cells. Data shown are combined from three independent experiments. H, adhesion of 4T1-RFP tumor cells to a monolayer of shNT or shEN 10T1/2 cells (H) or WT or ENKO mouse brain pericytes (I). Data show mean of adherent cells from three wells per condition at each time point. ***, P < 0.001. Equivalent results were obtained in an independent experiment. ns, nonsignificant.
(48, 49); however, analysis of tumors growing in WT or ENKO mice did not yield any significant differences in macrophage numbers, indicating that macrophages are not the driving force for endosialin-mediated tumor cell invasation (C. König and H.G. Augustin, unpublished data). In conclusion, although the mechanisms of tumor cell invasation are not well elucidated, these data indicate that the interaction of tumor cells with both pericytes and endothelial cells may play a role (50).

To delineate more directly the roles of individual cell populations, we mimicked the multicellular vascular crosstalk in a modified Transwell migration assay. Pericytes with or without endosialin expression were cocultured with endothelial cells, and tumor cells were analyzed for their ability to transmigrate across the endothelial layer. These in vitro reconstruction experiments validated the in vivo findings to show that endosialin expression by pericytes alone stimulated tumor cell transmigration. Importantly, the stimulatory behavior of endosialin-positive pericytes was independent of the effect of pericytes on endothelial cells as enhanced tumor cell transmigration was still observed in the absence of an endothelial monolayer. Moreover, this ability of endosialin-positive pericytes to promote tumor cell transmigration could not be recapitulated by pericyte-conditioned medium or pericyte-remodeled extracellular matrix, indicating a requirement for a direct interaction between tumor cells and tumor pericytes during invasation. While the detailed molecular mechanisms mediating this interaction await elucidation, analysis of global gene expression data of primary tumor cells isolated from WT and ENKO tumor-bearing mice indicate that endosialin-positive stroma does not alter the intrinsic tumor cell transcriptional profile (C. Viski and Clare M. Isacke, unpublished data), rather that it functions to modulate the local microenvironment. Indeed, it has been reported previously that recombiant endosialin can bind in in vitro assays to extracellular matrix components, including collagen and fibronectin (24), and in vivo to the vascular basement membrane (28). Thus, based on the available data, we hypothesize that endosialin does not function as an active metastasis promoting signaling factor in educating tumor cells to gain invasive behavior, but rather as a facilitator tethering tumor cells to the matrix and actively mediating their transmigration through the vascular basement membrane and underlying endothelial monolayer.

In conclusion, the data presented here causally link the expression of a metastasis-facilitating stromal receptor to a very specific step of the metastatic cascade and shed important mechanistic insight into the role of tumor-associated pericytes in mediating tumor metastasis. Importantly, the clinical relevance of these preclinical tumor experiments, involving independent tumor models with corresponding mechanistic cellular experiments could be validated by comparative pathology of human tumors. Indeed, analysis of human breast cancer samples revealed that the abundance of primary tumor stromal endosialin expression strongly correlated with metastatic progression and reduced recurrence-free survival. Consequently, the findings of this study and the unique oncofetal expression of endosialin warrant further consideration of endosialin as a therapeutic target to limit tumor progression and metastasis.

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

Authors’ Contributions
Conception and design: C. Viski, C. König, C.M. Isacke, H.G. Augustin Development of methodology: C. Viski, C. König, M.A. Kijewska Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Viski, C. König, M.A. Kijewska, C. Mogler, C.M. Isacke Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Viski, C. König, M.A. Kijewska, C. Mogler, H.G. Augustin Writing, review, and/or revision of the manuscript: C. Viski, C. König, M.A. Kijewska, C.M. Isacke, H.G. Augustin Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. König, H.G. Augustin Study supervision: C.M. Isacke, H.G. Augustin

Acknowledgments
We would like to thank Dr. David Huso (Johns Hopkins University, Baltimore, MD) for providing ENK0 mice. We gratefully acknowledge David Robertson, Marjan Iravani, Qiong Gao, Nicole Simonovich and the Histopathology Facility (Breast Cancer Now Research Center), and Maria Riedel (DKFZ) and Eva Besemfrier (DKFZ) for their assistance and support in this project. We would also like to thank the excellent technical support by the DKFZ Laboratory and Institute of Cancer Research Animal and Imaging core facilities.

Grant Support
This work was funded by Breast Cancer Now (C.M. Isacke), a Medical Research Council PhD studentship (C. Viski), grants from the Deutsche Forschungsgemeinschaft (SFBTR23 ‘Vascular Differentiation and Remodeling’ to H.G. Augustin) and the Helmholtz Alliance ‘Preclinical Comprehensive Cancer Center’ (H.G. Augustin). C.M. Isacke acknowledges NHS funding to the NIHR Biomedical Research Centre at The Royal Marsden and the ICR. H.G. Augustin is supported by an endowed chair from the Aventis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 8, 2016; revised July 7, 2016; accepted July 18, 2016. Published online September 15, 2016.

References
Activated Pericytes Promote Metastatic Dissemination


Endosialin-Expressing Pericytes Promote Metastatic Dissemination

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*Cancer Res* 2016;76:5313-5325.

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