



# Syndecan-1 facilitates breast cancer metastasis to the brain

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## Abstract

**Purpose** Although survival rates for patients with localized breast cancer have increased, patients with metastatic breast cancer still have poor prognosis. Understanding key factors involved in promoting breast cancer metastasis is imperative for better treatments. In this study, we investigated the role of syndecan-1 (Sdc1) in breast cancer metastasis.

**Methods** To assess the role of Sdc1 in breast cancer metastasis, we silenced Sdc1 expression in the triple-negative breast cancer human MDA-MB-231 cell line and overexpressed it in the mouse mammary carcinoma 4T1 cell line. Intracardiac injections were performed in an experimental mouse metastasis model using both cell lines. In vitro transwell blood–brain barrier (BBB) and brain section adhesion assays were utilized to specifically investigate how Sdc1 facilitates brain metastasis. A cytokine array was performed to evaluate differences in the breast cancer cell secretome when Sdc1 is silenced.

**Results** Silencing expression of Sdc1 in breast cancer cells significantly reduced metastasis to the brain. Conversely, overexpression of Sdc1 increased metastasis to the brain. We found that silencing of Sdc1 expression had no effect on attachment of breast cancer cells to brain endothelial cells or astrocytes, but migration across the BBB was reduced as well as adhesion to the perivascular regions of the brain. Loss of Sdc1 also led to changes in breast cancer cell-secreted cytokines/chemokines, which may influence the BBB.

**Conclusions** Taken together, our study demonstrates a role for Sdc1 in promoting breast cancer metastasis to the brain. These findings suggest that Sdc1 supports breast cancer cell migration across the BBB through regulation of cytokines, which may modulate the BBB. Further elucidating this mechanism will allow for the development of therapeutic strategies to combat brain metastasis.

**Keywords** Breast cancer · Brain metastasis · Syndecan-1 · Blood–brain barrier

## Abbreviations

BBB	Blood–brain barrier
Sdc1	Syndecan-1
MDA-231	MDA-MB-231
Sdc1 KD	Syndecan-1 knock-down

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NS1	Non-silencing sequence
Sdc1 OE	Syndecan-1 overexpression
EV	Empty vector
HER2+	Human epidermal growth factor receptor 2 positive
TNBC	Triple-negative breast cancer
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
BME	Basement membrane extract
NSG	NOD.Cg-Prkdc <sup>scid</sup> Il2rg <sup>tm1Wjl</sup> /SzJ
ANOVA	Analysis of variance
iHUVCEs	Immortalized human umbilical vein endothelial cells
HA	Human astrocytes
CI	Cell index
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
DAPI	4',6-Diamidino-2-phenylindole
GFP	Green fluorescent protein
PECAM-1	Platelet endothelial cell adhesion molecule-1
CM	Conditioned medium
GRO- $\alpha$	Growth regulated oncogene-alpha
ICAM1	Intercellular adhesion molecule 1
IL-8	Interleukin-8
IL-6	Interleukin-6
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
CCL5	C-C motif chemokine ligand 5
ELISA	Enzyme-linked immunosorbent assay
TMA	Tissue microarray
TCGA	The cancer genome atlas

## Introduction

The 5-year disease-free survival for breast cancer (BC) patients diagnosed with localized disease is 93–98%; however, for patients with distant-stage BC, e.g., brain metastasis, the survival is only 26–40% [1]. Women with brain metastases survive on average 2–16 months after diagnosis [2, 3]. Syndecan-1 (Sdc1) has been implicated in promoting breast cancer progression and is highly expressed in HER2+ and triple-negative breast cancers (TNBC) [4, 5]. Patients with TNBC or HER2+ breast cancer have a worse overall survival that is associated with increased metastatic burden [6].

Syndecans (Sdcs) are cell surface heparan sulfate proteoglycans (HSPGs) that act as co-receptors for growth factors, matrix proteins, cytokines, and chemokines. Through these interactions, the Sdcs mediate several biological functions, such as cell–cell and cell–matrix adhesion, proliferation, differentiation, migration, and angiogenesis. Many

have reported that the Sdcs play a dynamic role in cancer, whereby they can either promote or suppress cancer depending on the type of cancer and the type of Sdcs expressed [7–14]. Sdc1, typically expressed in epithelial cells, is widely detected in epithelial neoplasms derived from numerous origins [11, 15, 16]. It is specifically associated with poor prognosis in BC patients, and it has been linked to rapid BC growth rate [8, 17]. Up-regulation of mRNA and protein levels of Sdc1 have been detected in BC cell lines and patient biospecimens [4, 18]. Further, high levels of Sdc1 correlate with lower overall survival, relapse-free survival, distant metastasis-free survival, and worse metastatic relapse-free survival in BC patients [4, 7, 8, 19, 20]. Strong stromal expression of Sdc1 is a negative prognostic factor in BC and correlates with poor response to chemotherapy [11]. Taken together, these observations indicate that Sdc1 is associated with higher tumor grade, malignant forms of BC, and poor clinical outcome in patients [10].

Several in vitro and in vivo studies are congruent with the clinical expression of Sdc1 in BC. For instance, loss of Sdc1 suppresses Wnt-1-induced mammary carcinoma [21]. Moreover, Sdc1 is observed in stromal fibroblast and endothelial cells of invasive breast carcinomas where it is involved in tumor growth and angiogenesis [22]. Finally, Sdc1 directly participates in tumor cell spreading and adhesion, and through interaction with integrins, it promotes invasion [23, 24]. Notwithstanding this progress in understanding the diverse pro-malignant functions of Sdc1, little is known about its role in BC metastasis.

In the present study, we found that Sdc1 is important in mediating metastasis to the brain. Our findings indicate that Sdc1 acts by facilitating transmigration of BC cells through the BBB, but has no effect on attachment of BC cells to brain endothelial cells or astrocytes. Thus, we explored how Sdc1 supports this migration, and found that it affects the protein levels of a variety of cytokines/chemokines, many of which have been implicated in vascular permeability and cell migration. As such, our findings provide insight into how BC cells can invade and grow in the unique brain environment and the role that Sdc1 plays in this process. We believe this study will help with future development of therapeutic strategies to combat BC metastasis to the brain.

## Materials and methods

### Cell culture and reagents

The human TNBC cell line, MDA-MB-231 (MDA-231; gift from Dr. D. Welch, University of Kansas Cancer Center), was grown in DMEM/F-12 (ThermoFisher) supplemented with 5% FBS, L-glutamine (2 mM), sodium pyruvate (1 mM), and non-essential amino acids (0.02 mM, Gemini

Bio-Products). The mouse mammary carcinoma cell line 4T1 (gift from Dr. S. Spiegel, Virginia Commonwealth University [VCU]) was grown in RPMI 1640 (ThermoFisher) supplemented with 10% FBS (Gemini Bio-Products). BT549 cells were cultured in RPMI 1640 supplemented with 10% FBS and insulin (1  $\mu\text{g}/\text{mL}$ ). Immortalized human umbilical vein endothelial cells (iHUEVC, gift from Dr. W. Muller, Northwestern University [NU]) were cultured in human endothelial serum-free medium (Gibco) supplemented with 10% FBS, endothelial cell growth supplement (0.01  $\text{mg}/\text{mL}$ , Corning), and L-glutamine (2  $\text{mM}$ ). Human cerebral microvascular endothelial cells (hCMEC/D3 [hCMEC]; gift from Dr. B. Weksler, Cornell Medical College) were grown in endothelial basal medium-2 (Lonza) supplemented with 5% FBS, basic fibroblast growth factor (1  $\text{ng}/\text{mL}$ ), hydrocortisone (1.4  $\text{mM}$ ), ascorbic acid (5  $\text{mg}/\text{mL}$ ), and chemically defined lipid concentrate (1/100 dilution, ThermoFisher). All cell media contained penicillin (100  $\text{U}/\text{mL}$ ) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) (ThermoFisher). Primary cortical human astrocytes (HA) were grown on poly-L-lysine (0.02  $\text{mg}/\text{mL}$ , Bio-Techne)-coated dishes with HA media kit (ScienCell). All cells were maintained at 37 °C in a 5%  $\text{CO}_2$ /95% humidified air atmosphere and were routinely checked for mycoplasma. Cell lines were validated by Short Tandem Repeat analysis (DNA Diagnostic Center).

### Invasion and migration assays using the xCELLigence RTCA DP system

Invasion and migration assays were performed using 8- $\mu\text{m}$ -pore CIM (Cell Invasion/Migration)-Plates in the xCELLigence Real-Time Cell Analysis System (ACEA Biosciences) according to the manufacturer's recommendations. For invasion assays, the upper chambers of the CIM-Plate were pre-coated for 4 h with phenol red-free basement membrane extract (BME/Matrigel, Bio-Techne) (0.6  $\text{mg}/\text{mL}$ ) at 37 °C. MDA-231 NS1 and Sdc1 KD cell (100,000/well for migration; 20,000/well for invasion; seeded in 5% FBS-containing media) migration/invasion towards 20% FBS-containing media was measured for 24 h. The data were normalized in the xCELLigence software and results are reported as Mean Delta Normalized Cell Index (CI). High CI values are indicative of increased cell invasion and migration. These experiments were completed three times in quadruplicate. Statistical analysis, two-way ANOVA with Sidak's multiple comparisons, was performed.

### Animal experiments

All animal experiments were conducted in accordance with a protocol approved by NU and VCU Institutional Animal Care and Use Committee. For all injections, cell lines were collected using Versene (EDTA, ThermoFisher). The cells

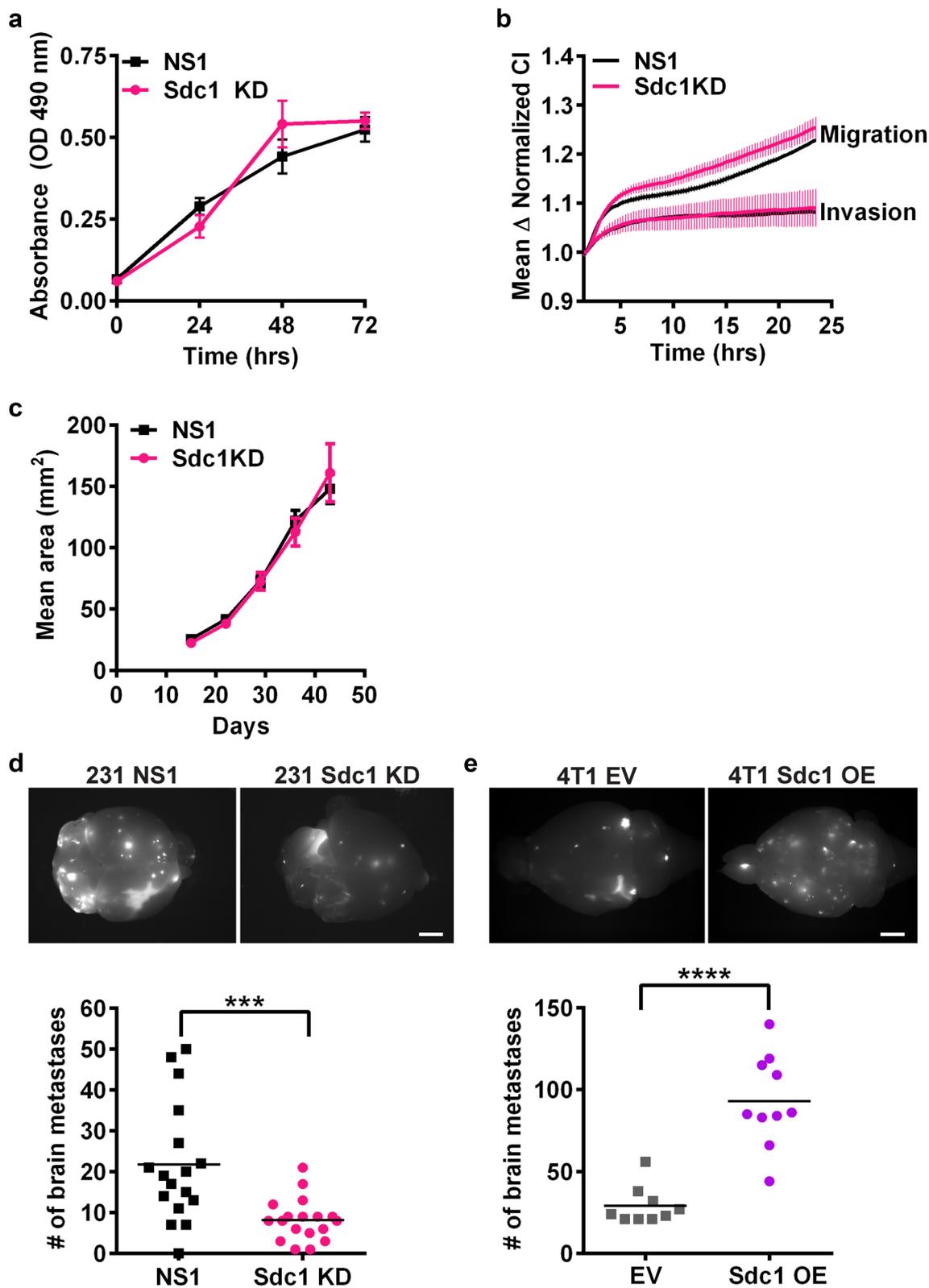
were mixed with phenol red-free BME (14.5  $\text{mg}/\text{mL}$ , Bio-Techne) for intraductal injections. The MDA-231 cell/BME mixture (100  $\mu\text{L}$ ) was injected into the lactiferous duct of the fourth mammary glands on both sides of either 6-week-old female athymic nude ( $0.5 \times 10^6$  cells/gland, Foxn1<sup>nu/nu</sup>, Envigo) or NSG mice ( $0.25 \times 10^6$  cells/gland, NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ, Jackson Labs) as described [25, 26]. Tumors were measured biweekly beginning 21 days after injection, and tumor area was calculated by width  $\times$  length ( $\text{mm}^2$ ). Entire primary mammary tumors were removed and weighed. Additionally, MDA-231 ( $2 \times 10^5$ ) or 4T1 ( $1 \times 10^5$ ) cells were injected into the left ventricle of the heart of 4–5-week-old nude, NSG, or Balb/cJ mice, respectively, as described [27]. Animals were sacrificed 21 days after injections. For both intraductal and intracardiac injections, all visceral organs, bones, brain, and lymph nodes were harvested for ex vivo examination of metastasis. To examine growth in the brain, the MDA-231 cells ( $1 \times 10^4$  cell/5  $\mu\text{L}$ ) were directly injected into the brain (0 Bregma, right 2.5 mm lateral, 3 mm deep) as described [28]. For all studies, visible metastases were imaged and counted using a Zeiss Stemi SV11 Apo fluorescent dissecting microscope. Metamorph analysis software was used to quantify brain metastases in cranial and caudal images. Statistics were determined by Student's unpaired t-test.

### In vitro transwell BBB assay

An in vitro BBB model was constructed as previously described [29]. Briefly, iHUEVCs were co-cultured with HA on opposite sides of an 8- $\mu\text{m}$ -pore PET FluoroBlok membrane transwell insert (BD Biosciences) for 3 days. Astrocytes ( $1 \times 10^5$  cells) were plated on the bottom side (day 1) and endothelial cells ( $5 \times 10^4$  cells) were plated on the upper side (day 2) of the membrane. Before seeding the cells, the membrane was coated with poly-L-lysine (HA, 0.02  $\text{mg}/\text{mL}$ , Bio-Techne) and gelatin (iHUEVC, 0.2%, Sigma). hCMECs were used in a similar format. After 2 days of iHUEVC/HA growth together, BC cells ( $1 \times 10^5$ /well) were seeded on iHUEVCs and migration towards BBB medium was assessed after 24 h [29]. Following fixation (4% PFA for 20 min), cells were stained with DAPI (167  $\text{ng}/\text{mL}$ ). The number of migrated tumor cells was analyzed from 6 images/well (Zeiss AxioVert 100 fluorescent microscope) using CellProfiler software. Statistics were determined by one-way ANOVA with Bonferroni multiple comparison post-test.

### In vitro brain section adhesion assay

The in vitro tissue section assay was performed as described [30]. Tumor cells were seeded onto fresh-frozen mouse brain (coronal), lung, or liver 20  $\mu\text{m}$  sections. After 90 min, cells



were fixed in 4% PFA, DAPI stained, cover slipped with ProLong Gold mounting medium (ThermoFisher), and imaged using fluorescence microscopy. The number of tumor cells

attached to each section was analyzed using ImageJ software. Statistics were determined by Student's unpaired t-test or one-way ANOVA with Bonferroni multiple comparison post-test.

**Fig. 1** Sdc1 expression facilitates breast cancer metastasis to the brain. **a** Silencing of Sdc1 expression does not affect proliferation of MDA-231 cells grown in serum-free media on BME/Matrigel. **b** Silencing of Sdc1 expression in MDA-231 cells had no significant effect on cell migration or invasion in vitro compared to MDA-231 NS1 cells.  $n=3$ . CI, Cell Index. **c** Top, representative fluorescent images of the cranial side of the brain after intracardiac injection of MDA-231 NS1 and Sdc1 KD cells in nude mice. Bottom, Mice injected with MDA-231 Sdc1 KD cells had a significant reduction in the number of metastases on the surface of the brain. NS1,  $n=17$ , Sdc1 KD,  $n=18$ .  $***p<0.001$ . Similar results were seen in NSG mice (data not shown). **d** Top, representative fluorescent images of the cranial side of the brain after intracardiac injection of control 4T1 EV and mSdc1 OE mammary cancer cells in Balb/cJ mice. Bottom, mice injected with 4T1 mSdc1 OE cells had a significant increase in metastases on the surface of the brain. EV,  $n=10$ , Sdc1 OE,  $n=18$ .  $****p<0.0001$ . **e** The growth rate of primary tumors arising from MDA-231 cells with reduced expression of Sdc1 was not significantly different from the control (NS1) cell lines, NSG mice. NS1,  $n=19$ , Sdc1 KD,  $n=18$ . Similar results were seen in nude mice (data not shown). For **a**, **b**, **e** Data point, mean  $\pm$  SEM. **c**, **d** Top, Bar, 2 mm

### Clinical samples and immunohistochemistry

Primary human breast tumors and BC brain metastases were obtained from the NU and VCU Pathology departments. Tissue microarrays (TMA) were made from 7 matched primary tumor and brain metastases from each institution and a total of 63 unmatched brain metastases (77 total). The tissue was obtained in compliance with protocols approved by the NU and VCU's Institutional Review Boards. Immunohistochemistry for Sdc1 (1:50 dilution of B-A38, Bio-Rad) was performed by the VCU Cancer Mouse Models Core Laboratory with the Leica Bond RX auto-stainer using heat-induced epitope retrieval buffer 1 (Leica, Sodium Citrate buffer, pH 6.0). Stained slides were then imaged on the Vectra Polaris (Akoya Biosciences). Immunoreactivity staining was evaluated and scored by clinical pathologists (MOI, JS, MS) in a blinded fashion.

TCGA level 3 gene expression data summarized as RSEM values were obtained using the TCGA2STAT R package v 1.2, along with the corresponding clinical annotations. The data were log<sub>2</sub>-transformed and analyzed using Kaplan–Meier curves and Cox proportional hazard model. Hazard ratio with 95% confidence intervals and logrank P values were reported. Each gene of interest was analyzed for its effect on survival by separating patients into high/low expression subgroups. A modified approach was used to estimate the best gene expression cutoff that separates high/low expression subgroups with differential survival [31]. The survival analysis was performed in R/Bioconductor statistical environment v.3.5.1 [32].

## Results

### Silencing of Sdc1 expression has no effect on cell proliferation and invasion in vitro

To investigate the role of Sdc1 in BC, we silenced its expression in MDA-231 cells (Sdc1 KD; 75% decrease both at the message and protein levels) as compared to the NS1 (control expressing non-silencing shRNAmir; Supplemental Fig. 1a). Of note, the other Sdcs were not significantly changed in Sdc1 KD cells [33]. For all experiments, Versene was used to collect cells to avoid cleavage of HSPGs from the cell surface, which occurs with trypsin [34]. Silencing of Sdc1 in MDA-231 cells had no effect on cell proliferation compared to NS1 cells irrespective of whether the cells were grown in serum or serum-free media (Fig. 1a; Supplemental Fig. 2a). Others have shown that loss of Sdc1 resulted in increased cell migration in keratinocytes and MDA-231 cells [14, 35, 36]. Indeed, in MDA-231 Sdc1 KD cells, we observed increased migration compared to NS1 cells using the xCELLigence System (Fig. 1b). However, there was no change in invasion through BME/Matrigel in the xCELLigence System (Fig. 1b). Similar results were observed using traditional transwell systems (data not shown). In conclusion, these experiments confirmed that loss of Sdc1 has no effect on MDA-231 cell proliferation and invasion, and only a slight increase in cell migration.

### Sdc1 mediates BC metastasis in vivo

To determine if Sdc1 has effects on BC primary tumor growth and metastasis in vivo, we injected both MDA-231 NS1 and Sdc1 KD cells into the fourth mammary fat pad of mice to allow for the formation of spontaneous metastasis. After approximately 60 days, the mice were sacrificed and the lymph node (LN), lungs, liver, brain, bone, ovary, and heart were collected to determine the percent of mice with metastases to these organs (Table 1). The primary tumor growth was not significantly different (Fig. 1c). Of all the organs assessed, differences in metastases following MDA-231 NS1 and Sdc1 KD cell injection were observed in only the liver (NS1: 37% vs Sdc1 KD: 17%) and the brain (NS1: 53% vs Sdc1 KD: 33%); however, these differences were not significant. Additionally, no differences were observed in other organs (Table 1). We hypothesized that the brain and bone metastases did not have sufficient time to grow out due to extensive lung metastases. Thus, we performed intracardiac injections (an experimental mouse model of metastasis) of MDA-231 NS1 and Sdc1 KD cells. Here, a significant decrease in the number of

**Table 1** Percent of mice (NSG) with metastases from the mammary gland to specific organs

	LN	Lung	Liver	Brain	Bone	Ovary	Days of growth	N
MDA-231 NS1	84	100	37	53	11	47	61	19
MDA-231 Sdc1 KD	88	100	17	33	11	50	59	18

LN lymph node, N number of mice per group; no significant differences determined by Fischer's exact test. Similar results were seen in the nude mice for lung, LN and ovarian metastases (data not shown); however, metastases to the liver, brain, or bone were not observed in nude mice as we have previously reported [25]

brain metastases was found in mice injected with MDA-231 Sdc1 KD cells as compared to mice injected with MDA-231 NS1 cells (Fig. 1d). No significant difference in metastasis to other organs was detected (bone, lung, liver; Supplemental Fig. 2b–d). To determine whether this effect could be seen in an immune competent animal, we used an allograft mouse model. We overexpressed Sdc1 (1.5-fold) in the 4T1 mouse mammary carcinoma cell line (Supplemental Fig. 1b) and intracardially injected these cells or EV (empty vector) control cells into the syngeneic BALB/cJ mice. We found that even this modest gain of Sdc1 expression resulted in significantly increased metastasis to the brain, bones, and liver (Fig. 1e; Supplemental Fig. 2e–g). These findings indicate that Sdc1 in tumor cells has no effect on primary tumor growth but plays a significant role in metastasis to specific organs, especially to the brain. We chose to focus on brain metastasis since a significant difference was observed in both the MDA-231 and 4T1 experimental metastasis models.

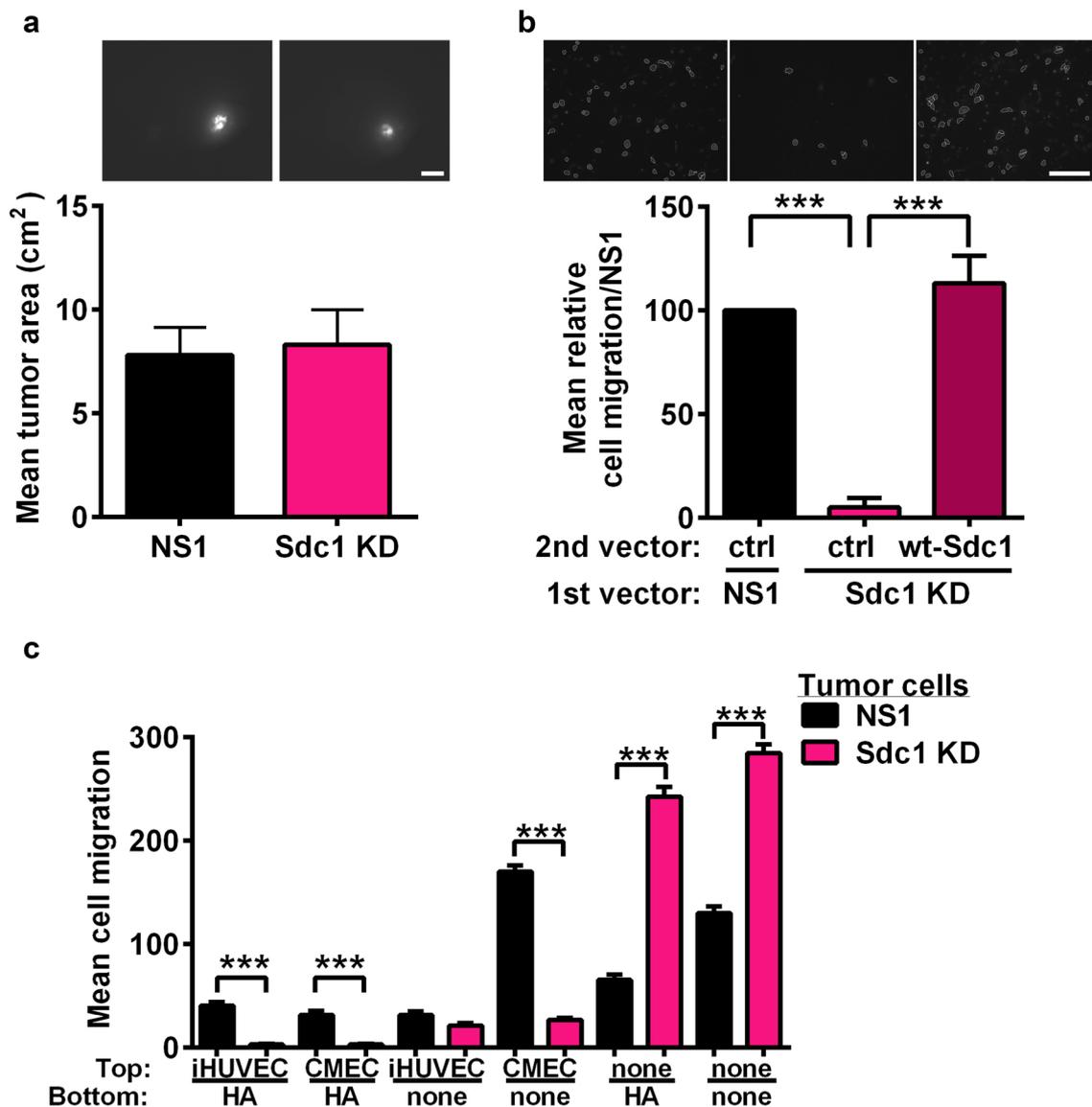
### Sdc1 facilitates BC cell transmigration across the BBB and attachment to perivascular regions in the brain

To examine whether Sdc1 affects tumor growth after seeding to the brain, mice were intracranially injected with MDA-231 NS1 and Sdc1 KD cells. No difference was found in the size of brain tumors detected (Fig. 2a). This suggests that Sdc1 may affect the ability of BC cells to extravasate and migrate into the brain environment, rather than to facilitate tumor outgrowth in the brain.

We next assessed the role of Sdc1 in BC cell transmigration across the BBB using a previously established in vitro BBB transwell model [29]. As confirmed in this model previously, we saw up-regulation of the glucose transporter 1 and  $\gamma$ -glutamyl transpeptidase 1 in the endothelial iHUVeC cells when co-cultured with HA, suggesting that iHUVeCs express BBB-specific proteins (data not shown) [29]. Furthermore, tight junction formation was observed by prominent ZO-1 staining (data not shown) [29]. We also used hCMEC brain microvascular endothelial cells in this same model (Fig. 2c) [29, 37]. When Sdc1 was silenced, there was a significant reduction in transmigration of the MDA-231 cells only across the in vitro BBB composed of

iHUVeC/HA, hCMEC/HA, or hCMEC alone compared to NS1 controls (Fig. 2b, c). Importantly, wild-type Sdc1 (wt-Sdc1; Supplemental Fig. 1c) can rescue the reduced BBB transmigration seen with Sdc1 KD cells (Fig. 2b). To confirm that the reduction in BBB transmigration was not due to an inability of the Sdc1 KD cells to adhere to the in vitro BBB, we seeded MDA-231 NS1 and Sdc1 KD cells onto a monolayer of either iHUVeCs, hCMECs, or HAs, and observed no difference in the attachment to these cells (Supplemental Fig. 3). Additionally, MDA-231 Sdc1 KD cells exhibited significantly more migration than NS1 controls through the insert alone as well as across HAs alone (Fig. 2c). This is similar to our xCELLigence data (Fig. 1b) and previous reports [14]. Taken together, these findings support the notion that Sdc1 facilitates BC cell transmigration across the BBB.

Carbonell et al. demonstrate that upon entry into the brain, cancer cells must first co-opt the vasculature to obtain nutrients and oxygen from blood circulation for successful colonization [30]. To further understand the ability of Sdc1 to support metastatic formation in the brain, we examined attachment to perivascular regions using an in vitro brain section adhesion assay [30]. MDA-231 NS1 cells exhibited significantly greater attachment compared to Sdc1 KD cells (Fig. 3a). This effect was rescued by expression of wt-Sdc1, and enhanced attachment was observed in NS1 cells with enforced expression of wt-Sdc1 (Fig. 3a). Additionally, immunofluorescence using PECAM-1 (CD31) to stain the brain vasculature demonstrated that cancer cells have a preference for attachment to the perivascular regions of the brain (Fig. 3b). The same brain section adhesion assay was also performed with BT-549 BC cells with silenced Sdc1 expression (Supplemental Fig. 1d) and 4T1 cells to confirm our results in other cell lines. We found that BT549 NS1 cells had significantly greater attachment compared to Sdc1 KD cells (Fig. 3c). Conversely, 4T1 Sdc1 OE cells displayed greater attachment to the brain section compared to EV control cells (Fig. 3d). To confirm that this difference in cell adhesion is specific to the brain, we performed a similar experiment using liver and lung sections and found no significant differences in attachment between MDA-231 NS1 and Sdc1 KD cells (Fig. 3e, f). Overall, loss of Sdc1 resulted in decreased attachment in an in vitro brain section adhesion assay, suggesting that Sdc1 may also facilitate



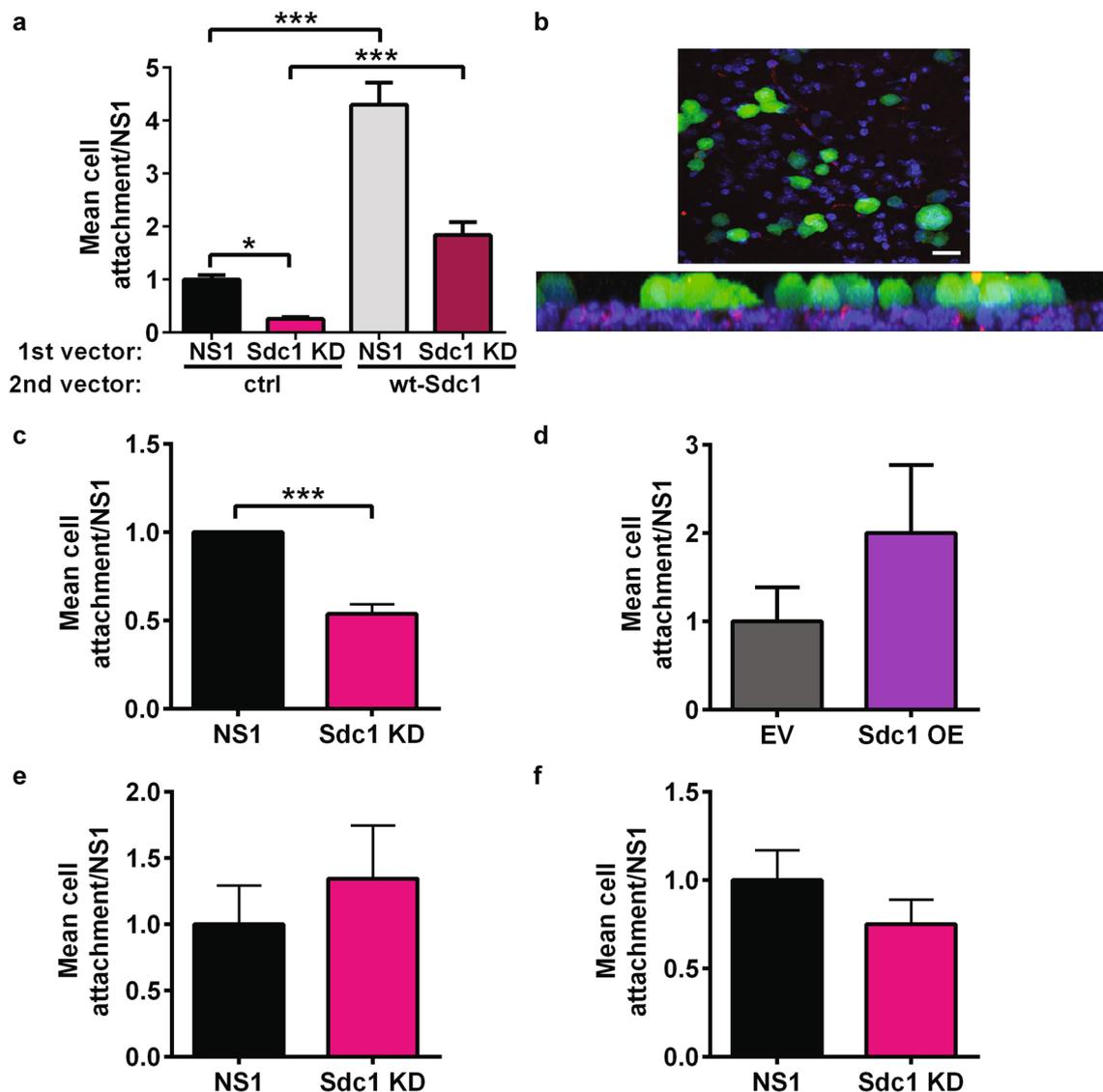
**Fig. 2** Sdc1 facilitates MDA-231 cell migration across the BBB. **a** Silencing of Sdc1 expression has no effect on BC growth within the brain when the cells were directly injected into the brain (intracranial injection). Top, fluorescent images of the cranial of the brain. Bottom, The mean tumor area  $\pm$  SEM was not different in mice injected with MDA-231 Sdc1 KD cells compared to those injected with MDA-231 NS1 cells,  $n=4$  for each group. **b** Representative images (top) and quantification (bottom) showing that silencing Sdc1 in MDA-231 cells results in decreased migration across an in vitro transwell BBB model system compared to NS1 control cells and MDA-231 Sdc1

KD cells expressing wt-Sdc1. The cells were transfected with either NS1 or Sdc1 shRNAmir (1st vector) and then were infected with either empty (ctrl) vector or wt-Sdc1 (2nd vector). Quantification of cell migration,  $n=4$ ,  $***p<0.001$ . **c** Silencing of Sdc1 expression decreases migration of the MDA-231 cells only when the cells are migrating through either hCMEC (CMEC) or a BBB made of iHUVVEC and HA or CMEC and HA. In contrast, silencing Sdc1 expression increases migration of MDA-231 cells across HA alone or no cells (none) compared to control NS1 cells.  $n=3$ ,  $***p<0.001$ . For all experiments, Bars, mean  $\pm$  SEM. **a, b** Top, Bar, 2 mm

brain metastatic formation through BC cell adhesion in the brain environment.

We predicted that Sdc1 signaling via its cytoplasmic domain might affect BC cell BBB transmigration and adhesion to the brain. Thus, we created various deletion mutants of Sdc1 cytoplasmic domain in the MDA-231 cell lines (Supplemental Fig. 1c and 4a). Wild-type Sdc1 as

well as the C2 domain deletion mutant are able to rescue the decreased MDA-231 BBB transmigration and attachment to perivascular regions when Sdc1 is silenced. However, a mutant Sdc1 lacking the entire cytoplasmic domain ( $\Delta$ C1VC2) is unable to rescue this decreased migration or attachment. Mutations at other regions in the cytoplasmic



**Fig. 3** Sdc1 facilitates breast cancer cell attachment to the perivascular regions of the frozen brain section. **a** Overexpression of wt-Sdc1 in MDA-231 NS1 control cells increased the ability of the cells to bind to a frozen mouse brain section, and expression of wt-Sdc1 in Sdc1 KD cells rescued the ability for those cells to attach to the brain section. The cells were transfected with either NS1 or Sdc1 shRNAmir (1st vector) and then were infected with either empty (ctrl) vector or wt-Sdc1 (2nd vector).  $n=3$ ,  $***p<0.001$ . **b** Representative image of MDA-231 NS1 GFP-positive cells (green) attach-

ing to perivascular regions (PECAM-1/CD31 positive staining for endothelial cells, red) in the brain (nuclei of brain cells DAPI stained, blue). Top panel,  $x$ -axis, Bar, 20  $\mu\text{m}$ . Bottom panel,  $z$ -axis of confocal image. Silencing of Sdc1 (Sdc1 KD) in BT549 BC cells (**c**) decreases attachment, and overexpression of mouse Sdc1 in 4T1 mammary cancer cells (**d**) increases attachment of these cells to perivascular regions in frozen mouse brain sections. Loss of Sdc1 has no effect on tumor cell attachment to frozen mouse liver (**e**) or lung sections (**f**). For all,  $n=3$ , Bars, mean  $\pm$  SEM.  $***p<0.001$

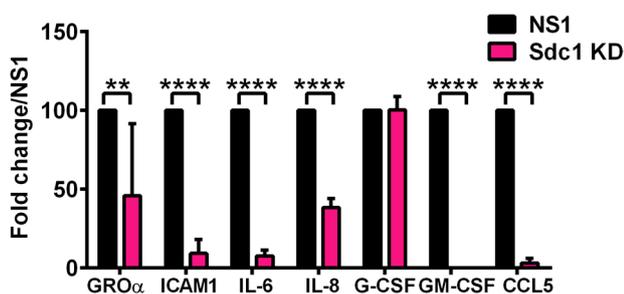
domain ( $\Delta V_{300-307}C2$ ,  $\Delta V_{296-307}C2$ ,  $\Delta VC2$ ) also significantly reduced the ability to rescue the reduced BBB transmigration and attachment compared to wt-Sdc1 rescue (Supplemental Fig. 4b, c). These findings demonstrate the importance of the cytoplasmic domain in BC cell BBB transmigration and adhesion and suggest Sdc1 intracellular signaling is essential for these cellular processes.

### Sdc1 regulates cytokines and chemokines secreted by BC cells

It is well established that secreted factors from cells promote BBB disruption to create a leakier barrier for cell entry into the brain [38–41]. Thus, we investigated whether loss of Sdc1 would drive changes to the BC secretome, which could conceivably result in increased BBB permeability and

BC cell adhesion to the perivascular regions in the brain. We specifically focused on cytokines and chemokines since they have been implicated in vascular permeability, inflammation, and cell migration, including transmigration across the BBB [40, 42–48]. Additionally, Sdc1 is known to interact with cytokines/chemokines, e.g., IL-6 and IL-8 [24, 49, 50]. Thus, to determine if Sdc1 affects BC cell cytokine/chemokine production and/or release, we performed a cytokine/chemokine array followed by a multiplex approach to confirm our results using 24-h conditioned medium (CM) collected from MDA-231 NS1 and Sdc1 KD cells. We identified differences in secreted cytokine/chemokine levels with a significant difference in GRO- $\alpha$ , ICAM-1, IL-6, IL-8, GM-CSF, and CCL5 (Fig. 4; Supplemental Fig. 5). Many of these cytokines/chemokines have previously been reported to have an impact on BBB integrity and/or facilitate BBB transmigration or affect the blood–tumor barrier, e.g., GRO- $\alpha$ , ICAM-1, IL-6, IL-8, GM-CSF, and CCL5 [45–48, 51]. Taken together, these findings indicate that Sdc1 plays a vital role in regulating cytokine and chemokine production and/or secretion from BC cells, and this may be important in supporting cancer cell entry into the brain.

We focused on IL-6 and IL-8 to determine if these factors could independently drive BBB disruption. These were selected based on reports demonstrating that they associate with Sdc1, can mediate vascular permeability, and IL-8 in particular can facilitate leukocyte transendothelial migration through binding to Sdc1 [24, 49]. Additionally, both IL-6 and IL-8 are found at the blood–tumor barrier [51]. We first determined the protein levels of IL-6 and IL-8 secreted by MDA-231 NS1 and Sdc1 KD cells using an ELISA. We found that 24-h MDA-231 Sdc1 KD CM contains significantly less (~50 pg/mL) IL-6 and IL-8 compared to NS1 CM (~150 pg/mL; Supplemental Fig. 6a, b). Using the xCELLigence System to examine BBB disruption, we added IL-6, IL-8, or a combination of both at a dose of 50 or 150 pg/mL. We did not observe any disruption except with the positive control (5% ethanol) (Supplemental Fig. 6c–e).



**Fig. 4** Silencing Sdc1 decreases cytokine/chemokine levels in MDA-231 cells. Quantification of human cytokines/chemokines from MDA-231 NS1 and Sdc1 KD cell 24-h CM using a multiplex assay. \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$

These results suggest that neither IL-6 nor IL-8 alone or in combination are sufficient to disrupt the BBB in this model system. Thus, we predict that BC-mediated BBB disruption is caused by multiple cytokines/chemokines.

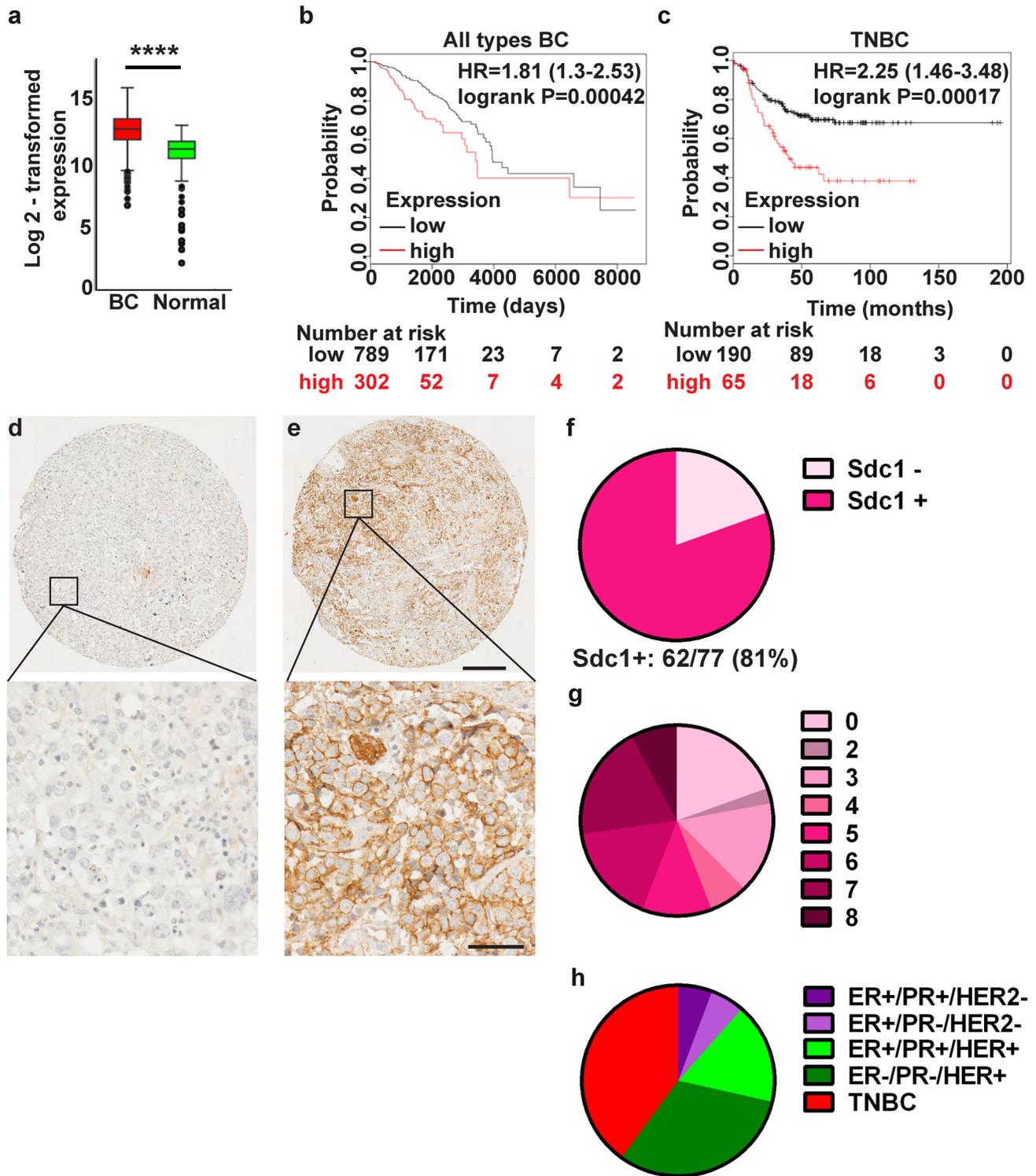
### BC patient brain metastases samples stained positive for Sdc1

To assess the clinical relevance of Sdc1 in BC and specifically in brain metastasis, we performed meta-analysis using The Cancer Genome Atlas (TCGA) and Kaplan–Meier Plotter [52]. TCGA analysis revealed a greater than four-fold increase in Sdc1 expression in BC versus normal breast tissue (Fig. 5a). Using Kaplan–Meier analysis of the TCGA data, we found that regardless of BC subtype, patients with high Sdc1 expression had worse disease-free overall survival than those with low Sdc1 expression (Fig. 5b). Focusing on the TNBC subtype, which is associated with an increased risk for brain metastasis, we found that patients with high Sdc1 have significantly worse disease-free overall survival compared to those with low Sdc1 expression (Fig. 5c). We also examined brain metastases samples from BC patients for Sdc1 protein expression using a TMA. Tissue samples were scored from 0 to 8 on the Allred scale. Sixty-two out of 77 (81%) tissue samples presented positive staining for Sdc1 with a score of 3 or above (Fig. 5d–g). The majority of these brain metastases arose from patients with either TNBC or HER2+ tumors (Fig. 5h). These findings demonstrate the clinical relevance of Sdc1 in brain metastasis and indicate that high Sdc1 expression is associated with worse prognosis and brain metastatic formation.

## Discussion

Sdc1 has been implicated in various cancers, including BC [4, 7, 8, 15, 18, 19, 21, 22, 53–58]. Previous reports have demonstrated that high Sdc1 expression on stromal fibroblasts and endothelial cells supports primary BC growth and angiogenesis and allows for an invasive-permissive micro-environment in a model-dependent manner [57–59]. Sdc1 expression on stromal fibroblasts is also required for efficient BC lung metastasis in the 4T1 mouse mammary carcinoma model [60]. In this study, we set out to determine if Sdc1 expression in BC cells plays a role in mediating metastasis. While we found that alterations in Sdc1 expression had no effect on BC proliferation or invasion in vitro (Fig. 1a, b), or primary tumor growth (Fig. 1c), we consistently observed effects on brain metastasis, regardless of the mouse model (Fig. 1d, e, Table 1). The findings we present here point to a novel role for Sdc1 in facilitating BC brain metastasis.

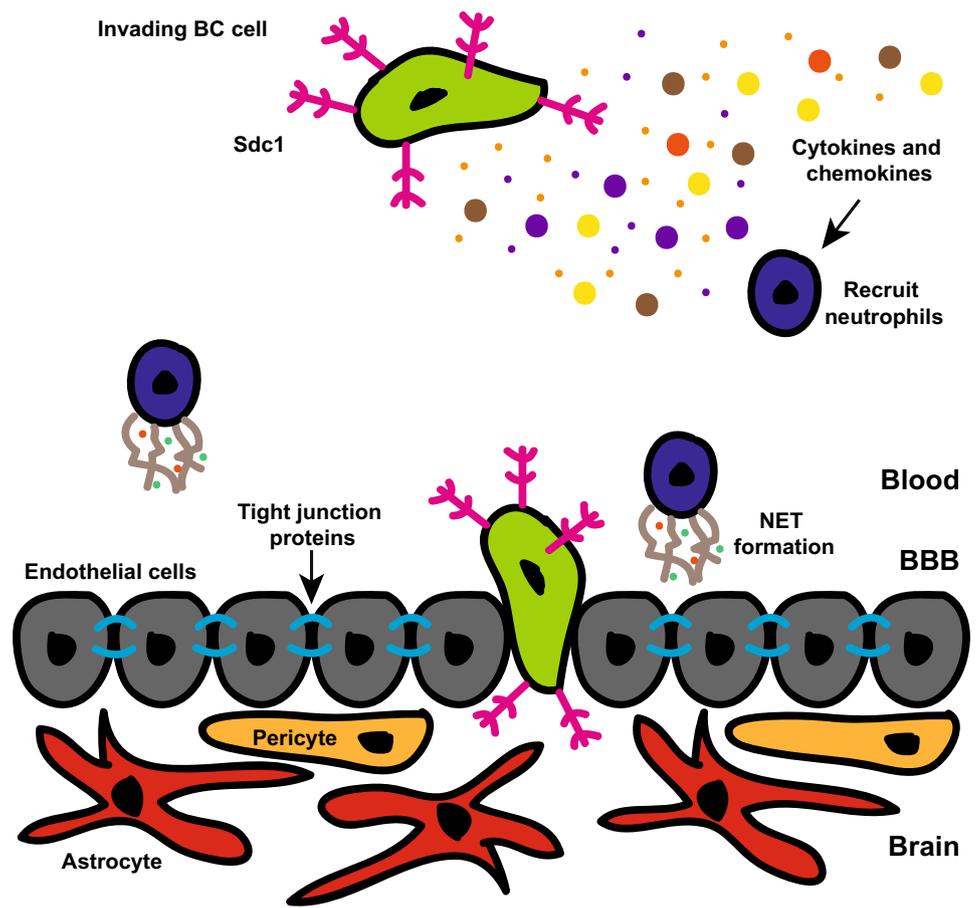
To seed the brain, metastatic cancer cells must cross a major obstacle, the BBB, a highly impenetrable physical and



**Fig. 5** Sdc1 is expressed in human breast cancer patient brain metastases. **a** TCGA data showing expression of Sdc1 are significantly different in cancerous versus normal breast tissue. \*\*\*\* $p < 2E-16$ . **b** Disease-free overall survival of BC patients of all subtypes is significantly different between patients with high and low Sdc1 expression.  $n = 1091$ ,  $p = 0.00042$ . **c** Disease-free overall survival of TNBC patients is significantly different between patients with high and low

Sdc1 expression.  $n = 255$ ,  $p = 0.00017$ . **d** Negative and **e** positive staining for Sdc1 in BC brain metastases TMA. Top, Bar, 200  $\mu$ m, Bottom, Bar, 50  $\mu$ m. **f** Percentage of BC metastatic brain samples that stained positive or negative for Sdc1. **g** TMA Allred scores (0–2, negative and 3–8, positive) for 77 patients. No patients received a score of 1. **h** Proportion of primary tumor subtype (for which this information was available) for each brain metastases present in the TMA

**Fig. 6** Working model. BC cells expressing Sdc1 produce and secrete high levels of various cytokines and chemokines. These factors are noted to play a role in BBB disruption. The chemokines can attract neutrophils and other leukocytes to facilitate transendothelial cell migration across the BBB. Neutrophils produce neutrophil extracellular traps (NETs), which induce BBB permeability. Overall, these factors may be key mediators of BC cell entry into the brain



metabolic barrier composed of endothelial cells, astrocytes, and pericytes, and then bind to the perivascular regions in the brain [61, 62]. Using an *in vitro* transwell BBB model system, we determined that Sdc1 facilitates BC cell transmigration across the BBB (Fig. 2b, c). This migration typically involves disruption of the BBB, particularly at the level of endothelial cell tight junctions as was shown for successful TNBC cell entry into the brain [38, 39, 41, 62, 63]. This disruption often occurs through secreted factors, e.g., cytokines/chemokines. Several groups have demonstrated that Sdc1 can regulate cytokines/chemokines, e.g., IL-6, IL-8, IL-34 [24, 49, 64]. Here, we found that Sdc1 can indeed regulate the production and/or secretion of cytokines/chemokines from BC cells, including those present at the blood–tumor barrier (Fig. 4, Supplemental Fig. 5) [51]. As described above, the cytokines/chemokines we identified have been reported to also affect BBB integrity and/or transmigration, e.g., ICAM-1, GM-CSF, CCL5, IL-6, and IL-8 [44–47]. There appears to be an intrinsic feedback loop between Sdc1 and cytokines/chemokines, which may be important in driving cytokine/chemokine production and/or release. For instance, Sdc1 is regulated by IL-6 and Sdc1 also regulates IL-6 and IL-8 [24, 49, 50, 65]. Thus, we hypothesized that IL-6, IL-8, or both in combination could

support BC cell transmigration across the BBB by promoting BBB permeability. However, in our *in vitro* model, IL-6, IL-8, or both together were not sufficient to mediate BBB disruption (Supplemental Fig. 6c–e). We predict multiple cytokines/chemokines may be involved in mediating BC entry into the brain, and identification of these cytokines/chemokines involved in disrupting BBB homeostasis could provide potential drug targets. It would also be important to consider how loss of Sdc1 signaling affects cytokine/chemokine production and/or release as we observed the inability of Sdc1 re-expression with mutated cytoplasmic domains to rescue BBB transmigration in MDA-231 Sdc1 KD cells. As we report, loss of the variable and C1 domains cannot rescue BBB transmigration and adhesion to the brain section (Supplemental Fig. 4b,c). This suggests that these domains are involved in cytokine/chemokine production and/or release. For future studies, we aim to identify the underlying mechanisms leading to brain metastasis facilitated by Sdc1.

Interestingly, while many cancers express Sdc1, this does not always correlate with increased risk for brain metastasis. For instance, prostate cancers overexpress Sdc1, and these cancers have a low incidence of brain metastasis (0.2–2%) [66–68]. Additionally, in cancers that do metastasize to the

brain such as lung cancer, it is unclear if Sdc1 correlates with poor or better prognosis [56, 69–72]. Furthermore, we previously showed that Sdc1 levels are similar in a normal breast cell line, MCF-10A, and across BC cell lines with various metastatic potential [33]. Overall, these findings indicate that Sdc1 does not independently drive cancer cells to metastasize to the brain but can facilitate this ability of the cells to cross the BBB. BC patients with high Sdc1 expression have worse overall prognosis, especially TNBC patients, and a high prevalence of BC patients' brain metastases express Sdc1 (Fig. 5b–g) [4, 7, 10]. The fact that Sdc1 is expressed on a large proportion of BC brain metastases correlates with our findings that Sdc1 is important in facilitating the ability of BC cells to cross into the brain. This suggests that metastatic BC patients with high levels of Sdc1 are at a greater risk for developing brain metastasis. Still, it would be interesting to also consider the role of Sdc1 in other brain metastatic cancers like melanoma. Brain metastatic melanoma is supported by astrocyte-secreted heparanase, and melanoma migration is promoted by heparan sulfate [60, 61]. Alternatively, other Sdcs may also facilitate brain metastasis. Sdc4 has been shown to play a role in brain metastatic BC cell invasion by affecting cytoskeletal dynamics through GEFH1 signaling; however, Sdc1 is not linked to this pathway [73]. Furthermore, unraveling the mechanism for Sdc1-supported brain metastasis could guide therapy development.

Therapies that target Sdc1 are currently being used in the clinic or in clinical trials. Zoledronate (an FDA-approved drug for multiple myeloma and bone metastases) acts by targeting various extracellular matrix components, including Sdc1, to inhibit cancer progression [11, 74]. Radioimmunotherapy to target Sdc1 showed promising results in mice with TNBCs expressing Sdc1 [5]. Indeed, targeting tumors with high Sdc1 using a Sdc1-antibody-DM4 cytotoxic drug conjugate, Indatuximab ravtansine, exert anti-tumor effects similar to trastuzumab emtansine [75]. These results demonstrate that Sdc1 is a suitable target for TNBC therapy, and these treatment options could be explored in reducing brain metastasis. Improving our understanding of Sdc1 expression in metastatic cancers could allow for a precision medicine approach to prevent brain metastasis.

## Conclusions

This study is the first to report that Sdc1 expression on BC cells facilitates brain metastasis. We propose a model in which Sdc1 fosters brain metastasis by supporting BC cell transmigration specifically across the BBB and provides evidence for Sdc1-regulated cytokine/chemokine production and/or release from BC cells (Fig. 6) [76, 77]. We predict that Sdc1 facilitates cancer cell BBB transmigration through

a mechanism involving multiple cytokines/chemokines, and plan to further investigate this in future studies.

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**Data availability** All data and materials presented in this study are available from the corresponding author upon request.

## Compliance with ethical standards

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

**Ethical approval** We declare that all work were performed in compliance with current U.S. laws and all animal experiments were conducted in accordance with a protocol approved by NU and VCU Institutional Animal Care and Use Committee. VCU IACUC Protocol AD10000943.

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