**Heparanase-Induced GEF-H1 Signaling Regulates the Cytoskeletal Dynamics of Brain Metastatic Breast Cancer Cells**

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**Abstract**

Heparanase is the only mammalian endoglycosidase which has been widely implicated in cancer because of its capability to degrade heparan sulfate chains of heparan sulfate proteoglycans (HSPG). Specifically, the cell surface HSPG syndecan-1 and -4 (SDC1 and SDC4) are modulators of growth factor action, and SDC4 is implicated in cell adhesion as a key member of focal adhesion complexes. We hypothesized that extracellular heparanase modulates brain metastatic breast cancer (BMBC) cell invasiveness by affecting cytoskeletal dynamics, SDC4 carboxy-terminal–associated proteins, and downstream targets. We used two independently derived human BMBC cell systems (MB-231BR and MB-231BR3), which possess distinct cellular morphologies and properties. Highly aggressive spindle-shaped 231BR3 cells changed to a round cell morphology associated with expression of the small GTPase guanine nucleotide exchange factor-H1 (GEF-H1). We showed that GEF-H1 is a new component of the SDC4 signaling complex in BMBC cells. Treatment with heparanase resulted in regulation of the SDC4/protein kinase C α axis while maintaining a constitutive GEF-H1 level. Third, GEF-H1 knockdown followed by cell exposure to heparanase caused a significant regulation of activities of Rac1 and RhoA, which are GEF-H1 targets and fundamental effectors in cell plasticity control. Fourth, L-heparanase augmented expression of β1 integrin in BMBC cells and of vascular cell adhesion molecule 1 (VCAM1; the major β1 integrin receptor) in human brain microvascular endothelial cells. Finally, using a newly developed blood–brain barrier in vitro model, we show that BMBC cell transmigration was significantly reduced in GEF-H1 knockdown cells. These findings implicate heparanase in mechanisms of cytoskeletal dynamics and in the cross-talk between tumor cells and vascular brain endothelium. They are of relevance because they elucidate molecular events in the initial steps leading to BMBC onset and capturing distinct roles of latent and active heparanase in the brain microenvironment.

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**Introduction**

Patients with brain metastatic breast cancer (BMBC) have an exceptionally poor prognosis, making BMBCs the most devastating and feared consequence of breast cancer. Despite increasing incidence of BMBCs and its recognition as a high clinical priority, mechanisms causing breast metastasis of breast cancer are understudied and remain largely unknown, limiting the effectiveness of therapeutic interventions (1–3).

Heparan sulfate proteoglycans (HSPG) are ubiquitous macromolecules located in the extracellular matrix and on the cell surface, consisting of a core protein and covalently attached heparan sulfate. Syndecans and glypicans are families of cell surface HSPGs whose members regulate the cross-talk between tumor and host cells by acting as coreceptors for heparan sulfate–binding factors. The syndecans are expressed by a family of 4 genes, SDC1–4 (4). Syndecans are type I transmembrane proteins oriented with an extracellular amino terminus and an intracellular carboxy terminus (4). Each of the SDC proteins has a unique extracellular and intracellular domain, thus allowing for distinct coreceptor phenotypes and intracellular signal transduction (4). Their functions place them at the center of cell signaling integration and are thus linked to tumor progression (5–8). Importantly, HSPGs are targets of heparanase (HPSE), the only mammalian endoglycosidase (endo-b-1→3-glucoaminidase), whose activity has been widely implicated in cancer metastasis (9, 10). Heparanase cleaves heparan sulfate at specific intrachain sites resulting in fragments (10–20 sugar subunits). These fragments are biologically active and can bind potent growth factors and angiogenic factors (9, 10). Of
interest, heparanase also has nonenzymatic functions in its unprocessed, latent 65-kDa form (L-HPSE), independent of its known endoglycosidase activity, which has been ascribed to the fully processed, active 58-kDa form of heparanase (a heterodimer consisting of 50 and 8 kDa subunits or A-HPSE; refs. 11–13). Furthermore, our laboratory has recently provided evidence of microRNA mechanisms suppressing BMBCs by directly targeting heparanase (14).

As tumor cells invade, they establish, abolish, and/or relocate transient focal adhesions. The invasive cell phenotype requires altered cytoskeletal dynamics, which are driven by the small GTPases: Rac1 and RhoA (15–20). The Rho-guanine nucleotide exchange factor-H1 (GEF-H1) is crucial in coupling microtubule dynamics to Rho GTPase activation in a variety of biologic settings. Importantly, GEF-H1 is a microtubule-binding protein which influences the dynamics of the actin cytoskeleton by inhibiting Rac1 and facilitating RhoA activity (21). Tumor cell extravasation requires a dynamically changing cell shape, which is associated with extensive cytoskeletal alterations. These, in turn, are driven by the small GTPases, Rac1 and RhoA, whose regulation is involved in tumor cell plasticity (19). Focal adhesion dynamics require Rac1 and RhoA activities with roles played by cell surface HSPGs as recent evidence indicates (19). Furthermore, we have reported that GEF-H1 differentially associates with SDC4 following cell exposure to purified preparations of human latent (L-HPSE) or active heparanase (A-HPSE; ref. 11). To negotiate with the blood–brain barrier (BBB), tumor cells often change their morphology inside the vascular lumen and form cytoplasmic protrusions which expand, but not disrupt, the vessel wall. These observations are of relevance in the pathology of brain metastasis (22) and require molecular deciphering.

We hypothesized that heparanase modulates HSPG-associated signaling in BMBC cells through mechanisms unrelated to its endoglycosidase activity. To this end, we used 2 human BMBC cell systems consisting of isogenic MDA-MB-231 variants which were independently selected in vivo. Both variants have increased ability to form brain metastasis (22) and require molecular deciphering. Consistent with the observations of relevance in the pathology of brain metastasis (22), we have tested (2011) for genomic DNA contamination, 1 μg of total RNA was digested with DNase I (Invitrogen) before first-strand synthesis. Two microliters of the inactivated DNase I digestion was used as template with a First Strand synthesis kit using SuperScript II reverse transcriptase according to manufacturer’s instructions (Invitrogen). The first-strand synthesis reaction was then diluted 1:1 with double-distilled water (ddH2O) to be used as single-strand cDNA template. PCR amplification was conducted in 20 μL reactions consisting of 1× AmpliTaq Gold buffer (Applied Biosystems), 2 mmol/L MgCl2, 300 μmol/L dNTP mix, 400 mmol/L primer pair, 2 μL single-strand cDNA template, and 2 U AmpliTaq Gold Taq polymerase (Applied Biosystems). PCR conditions were 94°C, 2 minutes; 40 cycles of 94°C, 20 seconds; 58°C, 15 seconds; 72°C, 42 seconds; and 72°C, 30 seconds. Gene accession numbers and DNA sequences for the oligonucleotide primer pairs used are shown in Supplementary Table S3. The PCR reactions were conducted using in a Mastercycler epgradient thermocycler (Eppendorf North America).

Materials and Methods

Cell culture

BMBC cells from 2 MDA-MB-231 cell systems independently derived from the in vivo selection of parental counterparts (231P and the brain metastatic variants 231BR and 231BR3 for brevity) were provided by Drs. Patricia Steeg (The National Cancer Institute, Bethesda, MD) and Janet Price (MD Anderson Cancer Center, Houston, TX). Cell lines were obtained between late 2009 and beginning 2010 and freshly recovered (<6 months) from liquid nitrogen before they were used for indicated experiments. The 231BR clones were obtained at early passage and tested (2011) for consistent in vivo abilities to metastasize to brain. Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 plus FBS (10%) and penicillin/streptomycin (1%), as published (14, 23). Human brain microvascular endothelial cells (isolate # 4 or HBMEC-4) were obtained following isolation of brain capillaries and their culturing as previously described (24, 25) and used at early passages. Human astrocytes were purchased from Lonza (Lonza Group Ltd.) and grown in media supplemented with AGM Bulletkit for astrocytes (astrocyte growth media, catalog no. CC-1423).

Reverse transcriptase-PCR

Total RNA was isolated from the cells using the RNeasy Plus Mini-Kit (Qiagen), according to manufacturer’s instructions. RNA yield was determined using a NanoDrop ND1000 spectrophotometer (NanoDrop products). To ensure lack of genomic DNA contamination, 1 μg of total RNA was digested with DNase I (Invitrogen) before first-strand synthesis. Two microliters of the inactivated DNase I digestion was used as initial template with a First Strand synthesis kit using SuperScript II reverse transcriptase according to manufacturer’s instructions (Invitrogen). The first-strand synthesis reaction was then diluted 1:1 with double-distilled water (ddH2O) to be used as single-strand cDNA template. PCR amplification was conducted in 20 μL reactions consisting of 1× AmpliTaq Gold buffer (Applied Biosystems), 2 mmol/L MgCl2, 300 μmol/L dNTP mix, 400 mmol/L primer pair, 2 μL single-strand cDNA template, and 2 U AmpliTaq Gold Taq polymerase (Applied Biosystems). PCR conditions were 94°C, 2 minutes; 40 cycles of 94°C, 20 seconds; 58°C, 15 seconds; 72°C, 42 seconds; and 72°C, 30 seconds. Gene accession numbers and DNA sequences for the oligonucleotide primer pairs used are shown in Supplementary Table S3. The PCR reactions were conducted using a Mastercycler epgradient thermocycler (Eppendorf North America).

Western blotting

Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and blocked with 5% (w/v) nonfat dry milk (for most antibodies) or 3% (w/v) bovine serum albumin (for anti-Rac1 or anti-RhoA antibodies) in TBS with 0.5% Tween-20 before being probed with appropriate antibodies. The antibodies and dilutions used in experiments were: SDC1 (1:1,000, clone B-A38) and Grb2 (1:1,000, 3972), purchased from Cell Sciences; SDC4 (1:1,000, ab24511) was from Abcam; full-length PKCo (1:1,000, #2506), GEF-H1 (1:1,000, #4076), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000, 14C10) were from Cell Signaling; β1 integrin (1:1,000, clone 656) was from BD Biosciences; Rac1...
(1:1,000, clone 23A8) and RhoA (1:500, clone 55) were from Millipore; and Tiam1 (1:1,000, sc872) and β-actin (1:1,000, sc-69879) were from Santa Cruz Biotechnology. Blots were washed with TBS containing 0.5% (v/v) Tween-20 in TBS (pH 7.4), before probing with horseradish peroxidase–conjugated secondary antibodies which were diluted in blocking solution (1:6,000, sc-2030 and sc-2031 from Santa Cruz Biotechnology). Blots were then exposed to film using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Glutathione-S-transferase pull-down analyses

Affinity chromatography by glutathione-S-transferase pull-down analyses (GST-PD) was conducted as previously described (26). Briefly, GST–SDC1 or GST–SDC4 fusion constructs in a pGEX-4T-3 vector (GE Healthcare) were transformed into the Escherichia coli strain BL21. Fusion protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 200 μM b-D-glucose (BBB extravasation assays were conducted on the basis of a protocol already described (27). Briefly, 6.5-mm Transwell membrane inserts with 8-μm pores (Corning) were treated on the underside with 100 μL of gelatin for 30 minutes, which was then gently removed. Astrocytes were then seeded to the underside of the inserts at a density of 1 × 10⁵ cells per well and allowed to grow in astrocyte growth media for 4 hours. The growth media were changed, and 1 × 10⁴ HBMECs were added to the top of each insert. The astrocyte-HBMEC–coated inserts were allowed to grow in astrocyte growth media for 3 days with fresh media daily. After 3 days, BMBC cells were treated with recombinant human heparanase (100 ng/mL; none, latent, or active) in DMEM/F12, 5% FBS, 10 mmol/L HEPES (pH 6.5) with penicillin/streptomycin (8) for 1 hour at 37°C and then trypsinized and counted. Cells were suspended at a density of 3 × 10⁵ cells: 300 μL DMEM/F12, 0.1% bovine serum albumin (BSA), then 300 μL of cell suspension was added to the tops of the inserts. Lower chambers contained HBMEC-conditioned media with 5 μmol/L N-formyl-Met-Leu-Phe (FMLP; Sigma). The cells were then left to invade for appropriate time points, then inserts were gently cleaned with Q-tips, and membranes removed and placed on slides for visualization (28). Red fluorescent protein expressing HBMEC cells were produced by stably transfecting cells with pmCherry-C1 (catalog no. 632524; Clontech) using FuGENE 6 Reagent (Roche Diagnostics). Procedures were repeated under similar conditions using GFP-labeled 231BR3 GEF-H1 short hairpin RNA (shRNA) knockdown clones. An equal number [1 × 10⁴] after fluorescence-activated cell-sorting (FACS) of viable GFP-labeled 231BR3 GEF-H1 shRNA knockdown cells were added to the top chamber at the start of invasion assays. Astrocytes and HBMEC cells were subtracted from quantification because we included control Transwell inserts with these 2 cell lines only, but no BMBC cells. An equivalent number of astrocytes and HBMEC cells was added to each assay, allowing for standardization/normalization of results. Furthermore, HBMEC cells were tagged (Texas Red fluorescence) to efficiently identify them for ease of analyses.

Immunofluorescence microscopy

Adherent cells (1 × 10⁵ cells per well) were grown on Lab-Tek II Chamber Slide w/Cover, 2-well size (Nalge Nunc Intl.). Cells were gently washed twice with 1 × PBS and fixed with 4% paraformaldehyde in 1 × PBS for 10 minutes. Then, cells were gently washed once with 1 × PBS, extracted with chilled extraction buffer consisting of 0.5% Triton X-100, 20 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 3 mmol/L MgCl₂, and 300 mmol/L sucrose on ice for 20 minutes. This was followed by two gentle 5-minute washes with wash buffer consisting of 0.1% NP-40 in 1 × PBS, cells were blocked with blocking buffer consisting of 5% horse serum and 0.1% NP-40 in 1 × PBS for 1 hour at room temperature (25°C). Cells were exposed to primary antibodies in blocking solution for 16 hours at 4°C, and then 3 gentle 5-minute washes with wash buffer were conducted. The cells were exposed to secondary antibodies in blocking solution containing Hoechst 33258 for 1 hour at room temperature (25°C). This was followed by 3 gentle 5-minute washes with wash buffer. The chambers were then removed from slides and mounting medium was placed directly upon stained cells, and coverslips were applied. The slides were cured 16 hours at 25°C and the coverslips were then sealed to the slides with nail polish. Antibodies and dilutions were: 1:500 VCAM1 (BBIG-V1 (4B2), BBA; R&D Systems), 1:100 GEF-H1 (C1 B4/7, HM2152, Hyclut Biotech), 1:400 tubulin (EP1332Y, ab52866; Abcam), 1:150 SDC4 (ab24511; Abcam), 1:50 PKCα (#2056; Cell Signaling Technology), 1:40 phalloidin (594; Invitrogen), and 2.5 μg/mL Hoechst 33258 mounting medium with antifade, ProLong (Invitrogen). Immunofluorescence images were acquired using a confocal microscope: laser source: X-Cite series 120, microscope: Zeiss Axio Imager.Z1, objectives: Plan-APoCHROMAT 63× or 100× 1.4 oil DIC, software version: AxiosVision 4.6.3. A consistent calibration of the confocal microscope, the presence of controls which were conducted in parallel, and the choice of fluorophore combination resulted in only a minimal overlap in wavelength which was readily compensated by the microscope.

In vitro BBB transmigration assay

BBB extravasation assays were conducted on the basis of a protocol already described (27). Briefly, 6.5-mm Transwell membrane inserts with 8-μm pores (Corning) were treated on the underside with 100 μL of gelatin for 30 minutes, which was then gently removed. Astrocytes were then seeded to the underside of the inserts at a density of 1 × 10⁵ cells per well and allowed to grow in astrocyte growth media for 4 hours. The growth media were changed, and 1 × 10⁴ HBMECs were added to the top of each insert. The astrocyte-HBMEC–coated inserts were allowed to grow in astrocyte growth media for 3 days with fresh media daily. After 3 days, BMBC cells were treated with recombinant human heparanase (100 ng/mL; none, latent, or active) in DMEM/F12, 5% FBS, 10 mmol/L HEPES (pH 6.5) with penicillin/streptomycin (8) for 1 hour at 37°C and then trypsinized and counted. Cells were suspended at a density of 3 × 10⁵ cells: 300 μL DMEM/F12, 0.1% bovine serum albumin (BSA), then 300 μL of cell suspension was added to the tops of the inserts. Lower chambers contained HBMEC-conditioned media with 5 μmol/L N-formyl-Met-Leu-Phe (FMLP; Sigma). The cells were then left to invade for appropriate time points, then inserts were gently cleaned with Q-tips, and membranes removed and placed on slides for visualization (28). Red fluorescent protein expressing HBMEC cells were produced by stably transfecting cells with pmCherry-C1 (catalog no. 632524; Clontech) using FuGENE 6 Reagent (Roche Diagnostics). Procedures were repeated under similar conditions using GFP-labeled 231BR3 GEF-H1 short hairpin RNA (shRNA) knockdown clones. An equal number [1 × 10⁴] after fluorescence-activated cell-sorting (FACS) of viable GFP-labeled 231BR3 GEF-H1 shRNA knockdown cells were added to the top chamber at the start of invasion assays. Astrocytes and HBMEC cells were subtracted from quantification because we included control Transwell inserts with these 2 cell lines only, but no BMBC cells. An equivalent number of astrocytes and HBMEC cells was added to each assay, allowing for standardization/normalization of results. Furthermore, HBMEC cells were tagged (Texas Red fluorescence) to efficiently identify them for ease of analyses.

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Rac1 and RhoA activity assays

BMBC cells were cultured in 0.5% DMEM/F12 with penicillin/streptomycin for 16 hours. The following day, the BMBC cells were treated with DMEM/F12, 0.5% serum, 10 mmol/L HEPES (pH 6.5) with recombinant human heparanase (100 mg/mL; none, latent, or active), and penicillin/streptomycin for 1 hour at 37°C. The 1-hour time was chosen because it aligns well with standardized heparanase activity determinations provided by the heparan sulfate–degrading enzyme assay (Takara Inc.; refs. 11, 28). Rac1 and RhoA small GTPase activity assays were conducted using the GLISA Kits (BK124, BK125; Cytoskeleton) according to manufacturer's instructions. Second, Rac1 and RhoA activities were normalized to total Rac1/RhoA protein content by conducting corresponding Western blotting. We determined the cell number to cell lysis buffer volume ratio that would reproducibly result in protein concentrations adjusted to 0.5 mg/mL. Cell lysates were then snap-frozen in liquid nitrogen to preserve GTP-bound GTTPase (Rac1 or RhoA) activity. GTTP-bound Rac1 GTPase was bound by Rac1-GTP–binding protein linked wells of 96-well plates. Bound active Rac1 was detected using a Rac1-specific antibody. The degree of Rac1 activation was determined by comparison with activity values obtained from lysates derived from untreated cells. Similarly, GTTP-bound RhoA GTTPase was bound by RhoA-GTP–binding protein linked wells of 96-well plates. Bound active RhoA was detected with a RhoA-specific antibody. Absorbance readings were obtained at 490 nm for both Rac1 and RhoA activities using a SpectraMax Plus 384 spectrophotometer (Molecular Devices). Both Rac1 and RhoA activity assays were conducted in quadruplicate and normalized to total Rac1 and RhoA within the same sample, as measured by Western blotting.

Knockdown of protein expression

The expression plasmids encoding shRNA to GEF-H1 and scrambled control were purchased from GeneCopoeia and transfected into 293Ta cells using manufacturer’s protocol. Supernatant containing lentiviral particles were then transduced into 231BR3 cells using 2 μmol/L polybrene (Millipore) and selected with 2.5 μg/mL puromycin (Sigma). Transduction efficiency was determined by GFP expression, and knockdown of GEF-H1 protein expression was confirmed by Western blotting. The precise target sequences can be found in Supplementary Table S1. The oligonucleotides encoding siRNA to heparanase and scramble control were purchased from Dharmacon (Thermo) and transfected into BMBC cells using manufacturer’s protocol. Knockdown of heparanase protein expression was confirmed by Western blotting. The precise target sequences can be found in Supplementary Table S2.

FACS

Fluorescently labeled (GFP) cell samples (scrambled, GEF-H1 shRNA 1/4 clones) were analyzed and sorted using the BD FACS Aria II 3 Laser High-speed Sorting Flow Cytometer equipped with 12 independent fluorescent channel capabilities and DIVA acquisition software. Each sample staining set included single-color controls to facilitate rigorous instrument setup and compensation. For each sorted sample, events were collected per list mode data file, gating and sorting for GFP-negative and GFP-positive cells (Supplementary Fig. S3). Cell were collected in 0.5 mL RPMI media (Invitrogen) and then used for culturing and visualization.

Cell adhesion assays

Fibronectin solution (Sigma: 0.5 μg/mL, 100 μL) was added to wells of a 96-well plate and allowed to coat the plate for 16 hours. Subsequently, the excess fibronectin solution was removed and 100 μL of HBMECs suspended at a concentration of 2 × 10^5 cells/mL in serum-free DMEM were added to the wells and allowed to adhere for 16 hours. HBMECs were then treated with recombinant human heparanase (100 mg/mL; none, latent, or active) for 1 hour at 37°C in DMEM supplemented with 10 mmol/L HEPES (pH 6.5), 5% FBS, and penicillin/streptomycin. The cells were then washed with PBS. BMBC cells (2 × 10^5 cells/mL, 100 μL) suspended in serum-free DMEM/F12 were added to the wells and allowed to adhere for 2 hours at 37°C. Wells were washed with 1 × PBS 3 times, and then 0.5% crystal violet solution was added (200 μL) to wells for 10 minutes. Subsequently, wells were washed with ddH2O 4 times and extracted with 200 μL 10% acetic acid for 10 minutes with gentle shaking. The extracted sample (560 nm) was then transferred to new wells, and absorbance readings were obtained (560 nm). Cell adhesion was determined by subtracting values for wells without BMBC cells from wells with BMBC cells.

Statistical analyses

Data were represented as mean ± SD. Significance values were obtained by the Student paired t test (*, P < 0.05; **, P < 0.01) when compared with untreated controls, unless otherwise indicated. All figures are representative of at least 3 independent experiments. Error bars in figures signify SD. P values < 0.05 were considered statistically significant.

Results

Differential regulation of BMBC cell adhesion, invasion, and GTPase activities by latent versus active heparanase

To test the hypothesis that GEF-H1 expression and the activities of Rac1 and RhoA are relevant in BMBC cells, we incubated 231P and the brain metastatic variant 231BR with human latent or active heparanase (L- or A-HPSE, respectively). Human latent and active heparanase properties, for example, molecular weight and heparanase enzymatic activity of forms, were verified (Supplementary Fig. S1; refs. 29, 30). Figure 1 shows a significant (P < 0.05) increase of Rac1 GTPase activity following exposure to heparanase in both 231P and 231BR relative to untreated control (none), particularly with the exogenous A-HPSE treatment (P < 0.01). The Rac1 activity response of the 2 cell lines to heparanase was similar. However, the effect of heparanase...
Differences were statistically significant from 231P to 231BR exposed to identical treatments, and in triplicate. Statistical comparisons via the Student paired content (Western blotting), respectively. GLISA assays were conducted for Rac1 and RhoA activities by GLISA assays normalized to total Rac1/RhoA protein content (Western blotting), respectively. GLISA assays were conducted in triplicate. Statistical comparisons via the Student paired t test were made from 231P to 231BR exposed to identical treatments, and differences were statistically significant. **, P < 0.01. B, Western blotting of total Rac1 and RhoA, GEF-H1, and associated signaling molecules following L- and A-HPSE cell treatments. Expression of Rac1-GEF Tiam1, known to be expressed in BMBC cells and regulated by c-src and FAK (29), and the adapter Grb2, which is a scaffold protein associated with these tyrosine kinases (30), is shown. Blots were probed for β-actin as a loading control. Refer to the Materials and Methods for additional details.

Figure 1. The modulation of Rac1 and RhoA GTPases by cell treatment with latent or active heparanase (L- and A-HPSE, respectively). Parental (231P) or brain metastatic (231BR) breast cancer cells were exposed to recombinant human L- or A-HPSE (100 ng/mL for 1 hour at 37°C), cell lysates were then prepared and analyzed accordingly. A, Rac1 and RhoA activities by GLISA assays normalized to total Rac1/RhoA protein content (Western blotting), respectively. GLISA assays were conducted in triplicate. Statistical comparisons via the Student paired t test were made from 231P to 231BR exposed to identical treatments, and differences were statistically significant. **, P < 0.01. B, Western blotting of total Rac1 and RhoA, GEF-H1, and associated signaling molecules following L- and A-HPSE cell treatments. Expression of Rac1-GEF Tiam1, known to be expressed in BMBC cells and regulated by c-src and FAK (29), and the adapter Grb2, which is a scaffold protein associated with these tyrosine kinases (30), is shown. Blots were probed for β-actin as a loading control. Refer to the Materials and Methods for additional details.

Heparanase Modulates Cytoskeletal Dynamics of Cancer Cells

on RhoA activity was quite different when comparing 231P and 231BR cells (Fig. 1A), as only the 231BR cells had significantly increased RhoA activity in response to exogenous treatment with either L- or A-HPSE (P < 0.01). Interestingly, heparanase (both latent and active) increased GEF-H1 expression in 231BR cells but not in parental 231P cells (Fig. 1B). We also investigated the expression of Rac1-GEF Tiam1, known to be present in BMBC cells and regulated by c-src and focal adhesion kinase (FAK; ref. 31), and the adapter Grb2, a scaffold protein associated with these tyrosine kinases (ref. 32; Fig. 1B).

For brain metastasis to occur, cancer cells must first adhere to the vascular endothelium, then cross the endothelial layer of the BBB, and finally invade and proliferate within the brain parenchyma. To assess heparanase involvement in cytoskeletal dynamics and cell extravasation, we developed a model to mimic the BBB, in which human brain endothelial cells (HBMEC-4) and astrocytes were cultured on opposite sides of the porous membrane of transwells (ref. 13; Fig. 2A). Next, we aimed to analyze roles of Rac1/RhoA activities and expression and BMBC cell transmigration using this in vitro coculture model (27). As initial investigations, we found differential effects on cell invasion and adhesion upon exposure of 231BR cells to recombinant heparanase (Fig. 2B and C). Exogenous L- or A-HPSE caused increased invasiveness in both 231P and 231BR cells (Fig. 2B), and knockdown of endogenous heparanase with siRNA reduced invasion of both 231P and 231BR cells (Fig. 2B). Both 231P and 231BR cells were also more adhesive in response to L- or A-HPSE (Fig. 2C). Of note, differential effects on cell adhesion versus invasion were detected in 231BR cells compared with 231P counterpart. While cell invasion was upregulated by both L- and A-HPSE (14), cell adhesion was predominantly mediated by the latent form of the molecule (L-HPSE; Fig. 2C). Interestingly, heparanase silRNA reduced adhesion below baseline only in 231BR but not the 231P cells (Fig. 2C). These data indicate a differential role for L- versus A-HPSE in adhesion and invasion of 231P and 231BR cells.

As integrins are important in regulating migration and invasion and may be engaged in BMBC–brain endothelial cell interactions as initial steps leading to brain metastasis, and because the vascular cell adhesion molecule 1 (VCAM1; α4β1 integrin is the major receptor of VCAM1; ref. 33) can function as a physical attachment site for a cell surface integrin/SDC4 complex, we hypothesized that extracellular heparanase may affect cell surface expression of VCAM1 in brain microvascular endothelial cells (HBMEC-4). Therefore, we analyzed HBMEC-4 for expression of VCAM1 in conjunction with the endothelial cell marker CD31 (control). We observed an overall increased staining for VCAM1 in response to exogenous L- and A-HPSE treatments which was most pronounced in a subpopulation of HBMEC cells (Fig. 2D and F). Importantly, we observed no change in morphology of HBMEC-4 following treatment with exogenous L- or A-HPSE (Fig. 2D). We analyzed heparanase and VCAM1 gene expression by RT-PCR: comparable levels of heparanase and VCAM1 gene expression were observed among cell lines. These levels were not significantly altered by exogenous L- or A-HPSE exposure (Fig. 2E). While mRNA of VCAM1 was not altered, VCAM1 protein expression was significantly upregulated by heparanase exposure (Fig. 2F).

These experiments suggest that the altered invasion of the breast cancer cells induced by heparanase may be, at least in part, due to the altered expression of VCAM1.
Heparanase alters the cell morphology in distinct BMBC 231BR cell systems

We sought to assess heparanase-mediated changes in cell morphology in the 2 isogenic BMBC cell systems associated with cytoskeletal dynamics, for example, alterations in the extent of polymerized actin and tubulin, the components forming actin stress fibers and microtubules, respectively (34).

The actin stress fibers among the MDA-MB-231-derived cell lines were strikingly different, as the fibers were thicker, longer, and more pronounced in 231BR3 cells, which also possessed a more spindle-shaped morphology (Fig. 3A and B). This contrasted with the 231P and 231BR cells which had a mixture of rhomboidal and spherical cell shapes (Fig. 3A and B). Of note, the distinctive cell morphologies by the 2 BMBC cell lines did not change during continuous passages (almost 20) in culture. Second, high basal levels of GEF-H1 in 231BR cells were observed to correlate with round/rhomboidal cell morphology. Interestingly, the exposure of 231BR cells to recombinant L- and/or A-HPSE resulted in 231BR cells changing morphology to a round cell shape associated with higher GEF-H1 expression (Fig. 3B). Conversely, networks of tubulin, an important cytoskeleton component (16–20), in these cell lines were mostly comparable; however, we detected tubulin which extended to the tips of the spindle-shaped 231BR3 cells (Fig. 3C). These results show that GEF-H1 is an important regulator of BMBC cell morphology that correlates with tubulin arrangement.

We conducted immunofluorescence staining of the breast cancer cells (231P, 231BR, and 231BR3) for GEF-H1, SDC4, and protein kinase C (PKC)α. First, we conducted co-immunostaining for GEF-H1, SDC4, and nuclei (Hoechst). We observed a robust GEF-H1 signal that co-localized with the SDC4 signal from the 231BR and the 231BR3 cells (Fig. 3D and E). Syndecan-4 was not only detected as a punctate pattern throughout the cell but also localized to small intracellular vesicles by a particularly intense immunodetection (Fig. 3D and E). Second, high basal levels of GEF-H1 in 231BR3 cells (Fig. 3C). These results show that GEF-H1 is a critical regulator of BMBC cell morphology that correlates with tubulin arrangement.

GEF-H1 is a critical regulator of BMBC cell GTPase activity

Because we identified a heparanase-induced modulation of the cytoskeleton which is closely associated with the morphology of human 231BR3 cells, we interrogated effects of knocking down GEF-H1 on Rac1 and RhôA activities. Cells (231BR3) were stably transduced with scramble control or 1 of 4 shRNA lentiviral constructs (clones 1–4; GeneCopoeia; Supplementary Tables S1 and S2), then expression of GEF-H1 and β1 integrin was investigated by Western blotting. We detected a significant decrease in GEF-H1 protein expression with a concomitant β1 integrin
expression, particularly for clones 1 and 4, which were used thereafter (Fig. 4A; Supplementary Fig. S2).

Next, to measure the effect of exogenous heparanase treatment upon the activities and expression of Rac1 and RhoA small GTPases, we treated stably transduced 231BR3 GEF-H1 shRNA cells with recombinant human heparanase (none, latent, or active). We measured significant increases of basal Rac1 activity in 231BR3 GEF-H1 shRNA clones 1 and 4 (12% and 15%; \( P = 0.0004 \) and \( P = 0.0003 \), respectively) compared with scramble control (Fig. 4B).

Notably, we observed little change in Rac1 or RhoA protein expression concomitant with shRNA knockdown of GEF-H1 expression (Fig. 4C). Treatment of the scramble control with A-HPSE increased Rac1 activity above basal Rac1 activity without changing Rac1 protein expression (Fig. 4B and C). Treatment with L-HPSE did not appreciably increase Rac1 activities in GEF-H1 knockdown clones above corresponding basal levels (Fig. 4B). Importantly, when 231BR3 GEF-H1 knockdown clones were exposed to A-HPSE, Rac1 activity was significantly reduced (−4% and −12%; \( P = 4 \times \)

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**Figure 2.** (Continued) D, heparanase treatment induces VCAM1 expression in CD31-positive human brain endothelial cells. Human brain endothelial cells (HBMEC-4) were grown in chamber slides and treated with human recombinant latent (L-HPSE) or active (A-HPSE) heparanase at 100 ng/mL for 1 hour at 37°C. Cells were then stained with antibodies raised against CD31 (red) or VCAM1 (green). Nuclei were stained with Hoechst 33258 (blue). Immunofluorescence and confocal microscopy were subsequently conducted and images taken using a 60× objective. E, analysis of heparanase and VCAM1 gene expression from human brain endothelial cells (HBMEC-4) by RT-PCR. Samples were analyzed for GAPDH gene expression control. Representative RT-PCR products: no exogenous heparanase treatment (None), treatment with L-HPSE (100 ng/mL for 1 hour at 37°C), treatment with A-HPSE (100 ng/mL for 1 hour at 37°C). Each set of RT-PCR reactions was carried out in triplicate. Refer to the Materials and Methods for additional details. pp, primer pair. F, analysis of VCAM1 protein expression and its regulation by L-HPSE and A-HPSE (100 ng/mL for 1 hr at 37°C) in HBMEC-4 cells by Western blotting. Blots were probed with β-actin as a loading control.
Figure 3. 231BR3 cell morphology is distinct from that of 231P and 231BR. A, brightfield images showing morphologies of 3 BMBC cell lines. Top, BMBC 231P cells; middle, BMBC 231BR cells; bottom, BMBC 231BR3 cells. Insets, representative images of cells at higher magnification. B, immunofluorescence staining for actin (red), GEF-H1 (green), and nucleus (blue) of 231P, 231BR (image mostly shows GEF-H1–overexpressing cells), and 231BR3 breast cancer cells. Cells were treated with human recombinant heparanase either in its latent or active form (100 ng/mL for 1 hour at 37°C). C, immunofluorescence staining for tubulin (red) and nucleus (blue). Arrows, tips of spindle in 231P, 231BR, and 231BR3 breast cancer cells. Controls refer to no heparanase treatment. Refer to the Materials and Methods for additional details. D, immunofluorescence staining for GEF-H1 (green, panels), SDC4 (green, insets), and nucleus (blue) for 231P, 231BR, and 231BR3 breast cancer cell lines. Cells were treated with human recombinant heparanase either in the latent or active form (100 ng/mL for 1 hour at 37°C). Control refers to no heparanase treatment. E, immunofluorescence staining for SDC4 (red, panels) PKCα (red, insets), and nucleus (blue) for 231P, 231BR, and 231BR3 breast cancer cell lines. Cells were treated with human recombinant heparanase either in the latent or active form (100 ng/mL for 1 hour at 37°C). Refer to the Materials and Methods for additional details.
10^{-4} and 1 \times 10^{-3}, respectively) which was not associated with decreased Rac1 expression (Fig. 4B and C).

As for RhoA, we measured reduced basal activity \( (P = 0.021 \text{ and } 3.1 \times 10^{-3}, \text{respectively}) \) when comparing the RhoA activities from scramble control versus the basal RhoA activity of the GEF-H1 shRNA knockdown clones, which was not associated with decreased RhoA protein expression (Fig. 4B and C). The RhoA activity of both GEF-H1 shRNA knockdown clones were further reduced subsequent to treatment with exogenous L-HPSE \( (P = 2 \times 10^{-4} \text{ and } 1.4 \times 10^{-3}, \text{respectively}) \) compared with the identical treatment of scramble control, again, not associated with decreased RhoA protein expression (Fig. 4B and C). Treatment of the scramble control with L- or A-HPSE maintained or slightly increased RhoA activity compared with untreated control (Fig. 4B). However, the RhoA activity was significantly decreased in response to A-HPSE treatment (32\% and 59\%; \( P = 2.04 \times 10^{-4} \text{ and } 4.26 \times 10^{-5}, \text{respectively} \)) in 231BR3 cells stably transduced to knockdown GEF-H1 expression, when compared with the RhoA activity from identical treatment of the scrambled control, which was not accompanied by reduced RhoA protein expression (Fig. 4B and C).

**GEF-H1 knockdown reduces cell invasion**

To form brain metastases, cancer cells must first negotiate with the BBB and implement mechanisms to cross this barrier and extravasate. We devised an in vitro assay to assess...
the invasive phenotypic changes that may occur as a result of knocking down GEF-H1 expression in the highly aggressive 231BR3 cell line. To aid in the visualization of the morphologic changes, we used GFP expression in conjunction with lentiviral GEF-H1 shRNA knockdowns and immunofluorescence microscopy (Fig. 5A–C; Supplementary Fig. S3). GFP expression by knockdown cells does not equate to shRNA expression due to stochastic factors. While shRNA is driven by the U6 promoter, GFP expression is driven by the cytomegalovirus (CMV) promoter. In addition, all GFP-tagged cells are puromycin-resistant cells.

Using our in vitro BBB transmigration assay, we found that the 231BR3 scrambled control cell line was highly invasive whereas both GEF-H1 shRNA 231BR3 clones were poorly invasive. Figure 5A–C shows images of underside of invasion chambers, visualizing the morphologies of invaded GFP-expressing 231BR3 cells whose number was quantified (Fig. 5D; see also Supplementary Fig. S4). Of note, the 231BR3 GEF-H1 shRNA knockdown clones had strikingly fewer GFP-expressing cells that had invaded through this in vitro BBB model (Fig. 5D). Also, while the morphology of the invaded shRNA scrambled control 231BR3 cells was...
primarily spindle-shaped, poorly invasive GEF-H1 shRNA clones were mostly rounded (Fig. 5A–C). The in vitro BBB transmigration assays were conducted in triplicate.

**GEF-H1 interactions with HSPG signaling components**

Because signal transduction emanating from the SDC4 intracellular carboxy-terminal (CT) domain of this HSPG component of focal adhesion is of relevance, we analyzed cell lysates from 231P, 231BR, and 231BR3 cells to determine whether similar associations could be identified in these cells. We identified a gradient of GEF-H1 expression (231P < 231BR < 231BR3; Fig. 6A). In addition, we determined the expression of GEF-H1 protein under normal conditions as well as following treatment with L- or A-HPSE. The expression of GEF-H1 was not modulated by heparanase (Fig. 6A). Conversely, heparanase treatment regulated the expression of PKCα differentially among the 3 cell lines. For example, we observed that treatment of 231P cells with heparanase induced PKCα expression, particularly in the presence of A-HPSE, whereas the opposite was true analyzing 231BR3 cells (Fig. 6A). The 231BR cell line had the highest detected PKCα expression whereas 231BR3 cells displayed highest levels of β1 integrin which was upregulated in the presence of heparanase cell exposure (Fig. 6A). Syndecan-4 was detected from each of the cell lines tested; however, its expression was not regulated by heparanase (Fig. 6A). Therefore, we investigated whether an association of GEF-H1 with SDC4 could occur in BMBC cells and, if so, whether the association would be modulated by treatment with exogenous heparanase. We used the intracellular CT region of SDC4 (Fig. 6B) in GST-PD analyses and detected a heparanase-regulated association of GEF-H1 with the intracellular SDC4 CT domain. The 231BR cells displayed an augmented SDC4 CT association subsequent to treatment with A-HPSE unlike 231BR3 cells, whose GEF-H1 association with the SDC4 CT decreased subsequent to treatment with A-HPSE (Fig. 6C). Analyses for PKCα interactions with the intracellular CT of SDC4 by GST-PD revealed that 231P and 231BR cells did show an association between PKCα and SDC4 CT whereas 231BR3 cells did not (Fig. 6D). Conversely, SDC1 CT did not possess the same association with GEF-H1 and PKCα as SDC4 CT in all 3 cell lines (Fig. 6C and D). These results show that GEF-H1 intimately associates with HSPG signaling components and is a target for regulation by heparanase.

**Discussion**

The work presented provides first-time evidence showing that (i) exogenous heparanase treatment of GEF-H1–expressing isogenic human BMBC cells altered cell adhesion and invasion; (ii) these phenotypic changes are dependent upon the type of exogenous heparanase treatment, for example, using the active versus the latent form of heparanase; (iii) heparanase-induced, GEF-H1–mediated effects altered Rac1 and RhoA activities and cytoskeletal dynamics; and (iv) an association of SDC4/PKCα and GEF-H1 may be relevant to govern these events (see also Fig. 7).

Figure 6. BMBCs show differential GEF-H1 and PKCα protein expression and heparanase treatment modulated SDC4 CT association. A, GEF-H1 and PKCα are SDC CT associated proteins in heparanase-treated BMBC cells. Whole-cell lysates from BMBC cells treated with L- or A-HPSE were immunoblotted for GEF-H1, PKCα, SDC4, or β1 integrin to determine protein expression levels in different BMBC cell lines. Blots were probed for β-actin as a loading control. Data presented are representative of 4 reproducible experiments. B, the production of GST fusion proteins. Affinity GST-PD for SDC1 and SDC4 were conducted as previously described (11). Eluted lysates (1 μg per lane) run on a 15% SDS-PAGE gel under reducing conditions. MW, molecular weight. C, GST-PD of GEF-H1 by SDC1/4 CT. D, GST-PD of PKCα by SDC1/4 CT. BMBC cells were treated with or without latent or active (L- or A-HPSE; 100 ng/mL for 1 hour at 37°C), then whole-cell lysates were generated, passed over GST-SDC1/4 CT fusion protein affinity columns followed by immunoblotting for GEF-H1 or PKCα as controls. Refer to the Materials and Methods for additional details.

Notions that cell surface syndecans are important to cell invasion and focal adhesion function and that Rac1 and RhoA are involved in tumor cell migration and phenotypic plasticity have been well-established (17, 18, 36). GEF-H1 is uniquely positioned to participate in this interplay as it binds and regulates these GTPases (37–39). Equally relevant, heparanase is long known to act as a potent protumorigenic, proangiogenic, and prometastatic enzyme (9, 10), and recent evidence implicates heparanase in cancer cell signaling.
Figure 7. A model for the proposed mechanisms involving heparanase-induced, GEF-H1–mediated regulation of Rac1 and RhoA activities in the cytoskeletal dynamics; and heparanase activities on SDC4 integrin as members of focal adhesion altering cell adhesion of BMBC cells.

independent of its enzymatic activity (11–13, 40). Here, we show that exogenous active heparanase (58 kDa), and even more the latent form of heparanase (65 kDa), can alter the cell morphology status and phenotypic characteristics in human isogenic BMBC cell systems, and expression of GEF-H1 in its abilities to affect Rac1/RhoA GTPases differentially. We noted the 231BR3 cell line to have a spindle-shaped cell morphology and high GEF-H1 expression than in 231P cells. Because cytoskeletal dynamics is a fundamental process for cell invasiveness, we hypothesized that upon reducing GEF-H1 expression in the brain metastatic 231BR3 breast cancer variant, the efficiency of invasion would be decreased, thereby reducing a core event of the metastatic process. Indeed, knocking down GEF-H1 expression reduced 231BR3 cell invasiveness; however, it also changed the morphology from a characteristic spindle shape to a spheroid type. The morphologic change of these GEF-H1 knockdown cell lines was revealed by visualizing invaded cells in our BBB transmigration assay. We consider this an indication that GEF-H1 may be involved maintaining the spindle shape morphology of the 231BR3 cells during invasive events. While the 2 MDA-MB-231–derived cell lines used here are isogenic, 231BR3 are distinct from 231BR cells from a morphologic perspective. Furthermore, 231BR3 cells differ from 231BR cells not only in the number of selection steps applied in vivo (3 vs. 6), in the cell injection route into animals (intracardiot vs. intracardiac), and the expression of EGFR receptor (EGFR) and HER2, 2 important biomarkers and hallmarks of BMBC; with 231BR3 cells expressing EGFR and HER2 (23) whereas 231BR cells having EGFR but very low HER2 expression (41).

We postulate that the spindle-shaped morphology presented by the 231BR3 cells is a result of an underlying cytoskeletal organization. We showed the spindles that are in polar opposition to each other are supported by actin stress fibers, known to be maintained by small GTPases Rac1 and RhoA whose activities are regulated by GTPase-activating protein (GAP) and GEFs (17, 21). Similarly, investigating heparanase-mediated alterations of tubulin, a key component of microtubules (16–20), was considered of relevance. Accordingly, we analyzed the expression of one specific GEF, GEF-H1. We discovered that GEF-H1 was expressed in each of the 3 MDA-MB-231–derived cell lines studied. Of note, GEF-H1 was highly expressed in MDA-231BR variants possessing high propensities to metastasize to brain.

The HBMEC-4 cells were tested for the expression of VCAM1, an important component for the formation of focal adhesions involving endothelial cells (42). Treatment with exogenous heparanase, particularly L-HPSE, induced VCAM1 expression in HBMEC-4 cells and that such increased expression was regulated posttranscriptionally. Interestingly, unlike the breast cancer cells we tested, the HBMEC-4 cells did not change to spheroid morphology in response to heparanase treatment.

Our data show that increased Rac1 activity correlates with decreased GEF-H1 expression as shRNA knockdown of GEF-H1 in 231BR3 cells resulted in increased Rac1 activity under basal conditions. This suggests that the increased Rac1 activity is likely due to removal of sustained Rac1 inhibition by GEF-H1 in 231BR3 cells. Furthermore, the increased Rac1 activity subsequent to exposing these cells to exogenous A-HPSE (scrambled control) was not measured from the GEF-H1 knockdown clones.

The decreased Rac1 activity we measured by comparing A-HPSE–treated scrambled control versus the GEF-H1 shRNA knockdown clone was not expected, considering that Rac1 expression was unchanged in the GEF-H1 knockdown clones and that GEF-H1 acts as inhibitor of Rac1 GTPase activity. This could be explained as knocking down GEF-H1 in 231BR3 cells results in the compensatory expression of another Rac1 inhibitor. Alternatively, this may involve a Rac1-inhibitory process that requires GEF-H1 but in a particular posttranslational state. Furthermore, GEF-H1 may inhibit Rac activities independent of Rac1, for examples, Rac2 and Rac3. However, Rac1 is the predominant form of Rac: analyses in our cells indicate that there is only very little Rac2 (which is hematopoietically specific; ref. 43) and Rac3 (unpublished observations; see also ref. 44), with GEF-H1 being the specific inhibitor of Rac1 (38). Second, the reduced RhoA activity detected when comparing the scrambled control with the 2 GEF-H1 knockdown cell lines, under all conditions tested, is likely attributed to reduced GEF-H1 expression as observed no decrease in RhoA protein expression and GEF-H1 was the only RhoA-GEF removed from the experimental conditions. This implicates GEF-H1 as a functional regulator of basal RhoA GTPase activity in 231BR3 cells and supports the idea that
exogenous heparanase can stimulate GTPase activity, but that the increased RhoA activity subsequent to heparanase treatment involves GEF-H1. Treatment with latent or active exogenous heparanase of 231BR3 cells stably transduced to constitutively express shRNA directed against GEF-H1 resulted in decreased RhoA activation, further supporting the notion that heparanase modulates RhoA activity via GEF-H1.

Third, we showed a potential mechanistic link for heparanase-regulated signal transduction to the cytoskeleton. This can be of relevance as it is known that the direct binding of PKCα to SDC4 CT increases its localization to focal adhesion sites (45). Focal adhesions are involved in cell migration and SDC4 is a known component of focal adhesions (37, 38). The carboxy terminus of SDC4 interacts with PKCα, which has also been implicated in cytoskeletal dynamics (46). Furthermore, PKCα and SDC4 provide mechanistic links to the extracellular environment for focal adhesion formation (47–50), and we have previously shown an association between the SDC4 CT domain and both GEF-H1 and PKCα using other cell systems (11, 51). Of note, SDC1 did not present the same association as SDC4 (Fig. 6C and D). Also, the combination of GEF-H1 knockdown and heparanase treatment downregulated cell invasiveness and altered Rac/Rho activity. This indicates that the 2 might act through a pathway originating with SDC4 and suggests that heparanase is an important regulator of cytoskeletal dynamics along with GEF-H1.

In conclusion, we have shown a GEF-H1 association with SDC4 using 3 breast cancer cell lines representing 2 BMBC models. We have shown a phenotypic morphology of spindle-shaped cells changing to a round cell shape during invasion as a result of GEF-H1 knockdown with consequent diminished cell invasiveness. Furthermore, we have shown that treatments with exogenous latent or active heparanase differentially regulate the GEF-H1/SDC4 association and activities of small GTPases Rac1 and RhoA as effectors of cytoskeletal dynamics. These findings are of relevance because they directly relate to the pathology of BMBC in its initial events leading to brain colonization, for example, interactions between BMBC and brain vasculature cells, and the formation of BMBC cytoplasmic cell protrusions and invadopodia stretching the brain microvessel wall for subsequent tumor cell extravasation (22). They substantiate roles for heparanase independent of its enzymatic activity, for example, acting as a cell adhesion molecule and signal transducer, and provide added impetus for developing inhibitors targeting latent heparanase and/or the use of heparanase inhibitors in novel cancer therapies, particularly in BMBCs.

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

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