Epidermal Growth Factor–Induced Heparanase Nucleolar Localization Augments DNA Topoisomerase I Activity in Brain Metastatic Breast Cancer

Lixin Zhang1, Peggy Sullivan3, Julie Suyama2, and Dario Marchetti1,2

Abstract

Identification of molecular mechanisms responsible for brain metastatic breast cancer (BMBC) is imperative to develop novel therapies. However, current understanding of the molecular circuitry that governs BMBC dissemination remains fragmentary. Heparanase (HPSE) is the only functional mammalian endoglycosidase whose activity correlates with cancer metastasis, angiogenesis, and the reduced postoperative survival of cancer patients, making it an active target for anticancer therapeutics. We hypothesized that human epidermal growth factor receptor 2 (HER2)/epidermal growth factor receptor (EGFR) activation promotes HPSE function in human BMBC. To address this, we examined HPSE content, activity, and intracellular trafficking in a HER2/EGFR-expressing BMBC model system and show that HPSE is present, functional, and correlates with HER2 status. Further, we showed that EGF induced nucleolar translocation of HPSE in these cells in a dose- and time-dependent manner upon activation of HER2/EGFR. Knockdowns of HER2/EGFR by small interference RNA abolished EGF-induced HPSE nucleolar translocation. It was also noted that nucleolar HPSE modulates DNA topoisomerase I (Topo I), an enzyme that is highly present in nucleoli, essential for DNA replication and transcription in a variety of tumors, and inhibited by heparan sulfate. Evidence is provided that HPSE can regulate Topo I activity, which subsequently affects BMBC cell proliferation. Finally, we showed that the nucleolar presence of HPSE with Topo I colocalization is detected only in HER2-overexpressing BMBC patient specimens. Altogether, these findings support the notion that HPSE is a critical downstream target of HER2 mechanisms driving BMBC and is potentially relevant for BMBC therapeutic interventions.

Introduction

Brain metastases are the most common intracranial tumors and their incidence is increasing (1). Despite the devastating consequences of brain metastasis for patient survival, mechanisms leading to brain metastasis are poorly understood. This is particularly valid in brain metastatic breast cancer (BMBC), whose patients have, even with best available treatments, only a 20% 1-year survival rate and a considerable associated morbidity (2). The identification of mechanisms responsible for brain metastasis is therefore imperative to improve current therapies and/or to develop more effective treatments.

Heparanase (HPSE) is the only functional mammalian endoglycosidase (endo-β-D-glucuronidase) degrading heparan sulfate (HS), the main polysaccharide component of growth factor–binding proteoglycans that are present on the cell surface and extracellular matrix (3-8). The relevance of HPSE in cancer invasion and metastasis has been established: in all human cancers investigated, HPSE upregulation correlates with metastasis, tumor angiogenesis, and the reduced postoperative survival of cancer patients (3). Overall, these findings indicate that this enzyme represents a promising target for anticancer drug development. HPSE inhibition is an area of intense investigation in cancer therapy with recent advances in clinical trials using HS mimics as HPSE inhibitors (3, 7, 8).

HPSE is a multifunctional effector that regulates various properties associated with tumor growth, angiogenesis, and metastasis (3, 8, 9). Similar to other enzymes, HPSE is first synthesized in a latent form that appears as a ~65-kDa protein when analyzed by SDS-PAGE. This protein lacks HS-degradative abilities (inactive HPSE); however, it possesses biological function (3, 8, 9). Latent HPSE subsequently

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undergoes proteolytic processing to yield the active enzyme (endo-β-D-glucuronidase activity) as a 58-kDa protein made up by two subunits, 50 and 8 kDa, respectively (3, 8).

Human epidermal growth factor receptor 2 (HER2) is a member of the epidermal growth factor receptor (EGFR) family (10). HER2 overexpression and EGFR positivity are key characteristics of BMBC (2, 10, 11). EGFR is important in HER2 signaling due to HER2/EGFR heterodimer formation (10). The correlation observed between HER2 overexpression and high BMBC incidence warrants investigations to elucidate mechanistic links between HER2/EGFR and BMBC determinants. Considering that our previous work has implicated HPSE as a molecular determinant of brain metastasis in murine and human melanoma models (4–6), we hypothesized that HER2 augments BMBC by inducing HPSE through HER2/EGFR activation by its ligand, EGF. To test this hypothesis, we examined the expression, intracellular trafficking, and activity of HPSE in a human, HER2-expressing, experimental BMBC model system (12). In this system, brain metastasis–selected variant cells were obtained after three cycles of sequential in vivo selection of MDA-231 parental (231P) cells following their injection into the internal carotid artery of nude mice and harvest of brain metastases. These metastasis–selected variants had increased propensities for brain metastasis and were named MDA-231BR1, MDA-231BR2, and MDA-231BR3 (231BR3 > BR2 > BR1; ref. 12).

Studies presented focus on EGF regulation of HPSE in HER2-containing BMBC through HER2/EGFR activation, HPSE trafficking as HER2 downstream target, and its effects on BMBC cell proliferation. First, we observed EGF-altered HPSE subcellular localization by detecting its expression, activity, and translocation to the nucleoli of BMBC cells, notably in the highly brain metastatic 231BR3 variant. This was abrogated by HER2/EGFR small interference RNA (siRNA) knockdown. Second, we discovered that DNA topoisomerase I (Topo I) is a target of EGF–induced nucleolar HPSE that enhances Topo I activity. It is known that Topo I is an enzyme that catalyzes changes in the superhelical duplex DNA state, is highly enriched in nucleoli (13), and is essential for gene transcription and stabilization of the mitotic machinery (14). Of note, Topo I is abnormally expressed in a variety of tumors, including brain neoplasms (14), and is inhibited by HS (15). Third, we found that EGF-induced HPSE modulated Topo I activity and BMBC cell proliferation. HPSE gene silencing or its inhibition resulted in an inhibition of Topo I activity and decreased cell proliferation. Finally, we show significant correlation between HPSE/Topo I nucleolar colocalization and HER2 status in human BMBC tissues.

Gene expression and functional analyses of BMBC cells and clinical samples have recently identified EGRF ligands, such as EGF; as mediators of BMBC (16). Our observations align with these findings and provide compelling evidence for roles of HPSE as a downstream target of EGF-activated HER2/EGFR and a player in mechanisms involving HER2 modulation to drive BMBC proliferation events.

**Materials and Methods**

**Antibodies and Reagents**

Rabbit anti-human HPSE polyclonal antibody (1453) and recombinant human HPSE preparations were kindly provided by Dr. Israel Vlodavsky (The Bruce Rappaport Faculty of Medicine, Technion, Israel; ref. 17). The monoclonal mouse anti-HPSE antibody was purchased from Cedarlane Labs. Both antibodies recognized the inactive (65 kDa) as well as active (50 kDa) forms of HPSE. Anti–EGFR, anti–phospho EGFR (Tyr1173), anti–DNA-Topo I, rabbit polyclonal anti-SC35, and monoclonal anti-fibrillarin antibodies were obtained from Santa Cruz Biotechnology Company. Anti–HER2 and anti–phospho HER2 (r877) were purchased from Cell Signaling Technology. For cell fractionation experiments, a monoclonal anti-integrin β1 antibody was used and purchased from Sigma. Secondary antibodies for immunofluorescence analyses were purchased from Invitrogen. The secondary antibodies were Alexa Fluor 488 donkey anti-goat IgG (H+L), Alexa Fluor 546 goat anti-mouse IgG (H+L), Alexa Fluor 488 goat anti-rabbit IgG (H+L), and goat anti-mouse Cy5. Secondary antibodies (goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP) used in Western blots were purchased from Santa Cruz Biotechnology. Laminarin sulfate (LS) was purchased from Sigma-Aldrich Corp. Cell proliferations kits were purchased from Promega, Inc.

**Cell Lines and Tissue Culture**

Human brain metastasis–selected MDA-231BR (BR1, BR2, BR3) and MDA-231 parental (231P) cells were obtained from Dr. Janet Price (Department of Cancer Biology, University of Texas MD Anderson Cancer Center, Houston, TX; ref. 12). The 231BR clones are the result of three cycles of injection into the internal carotid artery of nude mice with corresponding increased abilities to form brain metastasis in these animals (BR3 > BR2 > BR1; ref. 12). These clones were recently (2008) obtained at early passage and tested (2009) for their in vivo brain metastatic propensities. Cells were maintained in 5% CO2, 95% air at 37°C and tested (2009) for their in vivo brain metastatic propensities. Cells were obtained at early passage and tested (2009) for their in vivo brain metastatic propensities. Cells were maintained in 5% CO2, 95% air at 37°C using DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin antibiotics (Invitrogen).

**Tissue and Slide Preparation**

Twenty-eight consecutive cases of metastatic breast carcinoma to brain and paired tumor primary were retrieved from 2001 to 2009 at a single institution (University of California at Los Angeles Medical Center, Los Angeles, CA) using the pathology computer database search engine (Tamtron). Original glass slides were re-reviewed by one of the coauthors (PS) who is a breast cancer pathologist. The diagnosis of BMBC and HER2 expression status were confirmed for each case and blocks containing the greatest amount of carcinoma were selected for immunohistochemical analyses. Archived, formalin-fixed, and paraffin-embedded tissue blocks were retrieved, sectioned at 4-μm
intervals, and placed on glass slides. The presence of metasta-
static carcinoma was confirmed by reviewing the H&E slide.
Metastatic breast carcinoma to brain was characterized mor-
phologically. Adjacent viable brain parenchymal tissue was
detected in most H&E sections, showing changes consistent
with reactive gliosis, including an increase in glial cell num-
ber and gemistocystic change.

RNA Extraction and Real-Time Quantitative PCR
(RT-PCR)
Total RNA was extracted from indicated cells using Trizol
(Invitrogen) reagent. SYBR Green (Thermo Scientific)
RT-PCR was performed using a 1-Cycler detector (Bio-Rad
Laboratories, Inc., Hercules, CA) according to the manufac-
turer’s protocol. The sequences of primers used for detection
of HPSE gene cDNA were: 5′-GTTCTAATGCT-
CAGTTGCCCT-3′ and 5′-ACTGCCACCCATTGAT-
GAAA-3′; for the control L19 ribosomal RNA gene were:
5′-CTGAAAGTCAAGGGATGTG-3′ and 5′-GGACA-
GAGTTCTGATGATC-3′. Primer concentration and
PCR conditions were optimized. Each cDNA (50 ng) was
amplified using 0.5 mM of specific primer under these cycle
conditions: 95°C for 10 min, followed by 40 cycles of 94°C
for 15 s and 60°C for 1 min. A standard curve was generated
for each gene. Additionally, a dissociation curve was drawn
after each experiment to confirm that a single product was
amplified. The comparative CT method was used for quan-
tification of HPSE gene expression.

Western Blotting
Cells were serum starved overnight (16 h) at 37°C, then
stimulated with EGF (E; Sigma) or serum-free medium
only (S) as indicated in figures legends before being lysed
using lysis buffer (Cell Signaling Technology). Total lysates
were then resolved by SDS-PAGE (50 μg per lane) and
transferred to nitrocellulose membranes. Following standard
procedures, Western blot analyses were done using mouse
anti-fibrillarin antibody, or rabbit polyclonal/monoclonal
mouse anti-HPSE antibodies, anti-EGFR and anti-phospho
EGFR (Tyr1173), and anti-HER2 and anti-phospho HER2
(Tyr877). Protein levels were analyzed by densitometric
scanning using QUANTITY ONE program (Quality Asso-
ciates International, Inc.).

Immunostaining and Confocal Microscopy
Cells (231P and 231BR3) were grown on coverslips in
12-well plates and serum starved for 16 h before treatment
with EGF (100 ng/mL, 30 min or as indicated). Cells were
then fixed with 4% formaldehyde in PBS, permeabilized
with 0.1% Triton X-100, and blocked in 10% normal goat
serum followed by overnight (16 h) incubation at 4°C with
the specific primary antibody (anti-HPSE or as indicated),
diluted 1:200 in blocking buffer, followed by secondary
antibody (1:400 dilution in blocking buffer) incubation for
1 h at room temperature (25°C) and processed as described
in figures legends. Nucleolar localization was confirmed by
using an antibody to fibrillarin, which is a specific nucleolar
marker (18). Nuclei were counterstained with propidium
iodide (PI). Stained cells were then analyzed using confocal
microscopy (LSM 510 model, Carl Zeiss, Inc.).

SiRNAs and siRNA Transfections
Knockdown studies applying siRNA technology were
done using ON-TARGETplus SMARTpool siRNA re-
agents (Dharmacon). SMARTpool for each molecule
contained four nonoverlapping siRNA oligos. Specific siRNAs
used include human ERBB2/HER2, EGFR, and HPSE.
Human 231BR3 cells growing at 70% to 80% confluency
were transfected using the FuGene HD transfection reagent
(Roche Applied Science) and with SMARTpool siRNAs
(100 nmol/L for 48 h) for HER2, EGFR, and/or HPSE,
per the manufacturer’s procedures. In all transfections, con-
trol siRNAs (nontarget siRNAs) were used for siRNA spec-
ificity control (siCTRL). The efficiency of siRNA
knockdown was confirmed by Western blotting analyses
24 to 48 h after transfection.

Cell Fractionation and Isolation of Nucleoli
Human BMBC (231BR3) cells were treated with EGF
(100 ng/mL) for 30 min, followed by cell fractionation
to isolate cytoplasmic, nuclear, and nucleolar fractions.
Nuclear fraction was separated from the cytosol using
NE-PER nuclear and cytoplasmic extraction reagents
(Pierce) following the manufacturer’s instructions. Cell nu-
cleoli were isolated following a published procedure (19).
Briefly, cells were grown to ~70% confluency and fixed by
incubation with 0.2% of formaldehyde for 10 min at room
temperature (25°C). After being washed with PBS, cells were
then harvested and nucleoli were released by sonica-
tion on ice (two to three bursts of 10 s each) using a Sonic
Dismembrator, Model 100 (Fisher Scientific) equipped
with a fine probe. Following sonication, nucleoli prepara-
tions were pelleted by centrifugation (20-30 s at 15,000 g),
resuspended in a LM buffer [10 mmol/L HEPES (pH 7.5),
0.88 mol/L sucrose, 1 mmol/L MgCl2, 1 mmol/L DTT, and
a cocktail of protease inhibitors], and repelleted. Sonicated
nuclei were monitored under a phase-contrast microscope
with nucleoli readily observed as dense, refractile bodies.
Isolated nucleoli were stored at ~80°C and used for subse-
quent analyses. To verify fraction purity, specific markers
(20) and related antibodies used were as follows: monoclonal
anti-integrin β1 antibody, polyclonal rabbit anti-SC35,
and monoclonal anti-fibrillarin antibody for cytoplasmic/cell
membrane, nuclei, and nucleoli preparations, respectively.

HPSE Activity Assays
HPSE enzymatic activity was examined using a com-
mmercial HS degrading enzyme assay kit (TaKaRa Mirus
Biomedical, Inc.; ref. 19). The kit functions on the principle
that HS loses its ability to bind to basic fibroblast growth
factor when degraded by a HS-degrading enzyme. Biotiny-
lated HS is used as a substrate for the enzyme. The unde-
graded substrate bound to fibroblast growth factor is then
FIGURE 1. Expression and induction of HER2 and HPSE in 231BMBC cells. HPSE expression correlates with endogenous HER2 content and brain metastatic propensities of 231BR variants, and is augmented by EGF treatment. A, Real-time PCR (RT-PCR) analysis of HPSE transcript levels in human BMBC cell lines (231BR1, 231BR2, 231BR3) and parental counterparts (231P). Total RNA was extracted from each of these lines and HPSE gene expression was determined by RT-PCR, and normalized to corresponding controls as described in Materials and Methods. B, Western blot analysis of HER2 and HPSE levels in MDA-MB 231 parental (231P) and 231P-selected BMBC variant cell lines (BR1, BR2, and BR3). Cell lysates were prepared using lysing buffer (Cell Signaling Technology), and total proteins (50 μg) were loaded on 10% SDS-PAGE, transferred to nitrocellulose membrane, and incubated with anti-HPSE (1453) or anti-HER2 antibodies as described in Materials and Methods. β-Actin was used as loading control. C, HPSE activity was examined using the HS degrading enzyme assay kit (TakaRa Mirus Biomedical, Inc.) in the three BMBC 231BR variant lines and parental 231P. All samples and readings were done in triplicate. Activity is detected as the inverse of decrease in absorbance (450 nm), per kit instructions (20). Columns, mean of three independent sets of experiments with each experiment done in triplicate; bars, SD. *, P < 0.01. D, EGF-induced HER2 and EGFR phosphorylation and HPSE expression. Cell lysates were prepared and analyzed by Western blotting as in B. Indicated cells were treated with EGF (E; 100 ng/mL for 30 min at 37°C) or without the factor following overnight (16 h) serum starvation (S). Fifty micrograms of total proteins from the cell lysates were then loaded on 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-HPSE antibody (1453). After being stripped with the Restore Western Blot Striping Buffer (Thermo Scientific), the blot was probed using antibodies for indicated proteins. β-Actin was used as loading control. E, cells (231P and 231BR3) were treated with EGF or without (control) as per D, then HPSE activity was examined using the HS degrading enzyme assay kit (TaKaRa Mirus Biomedical, Inc.). Columns, mean of three independent sets of experiments with each experiment done in triplicate; bars, SD. **, P < 0.001.
FIGURE 2. EGF induces HPSE nucleolar localization in human BMBC cells. A and B, Western blotting analyses for EGF treatment of HPSE levels compared with fibrillarin staining as specific nucleolar marker (18). Human BMBC (231BR3) cells were serum starved for 16 h at 37°C and cell nucleoli were isolated as described in Materials and Methods. Nucleoli were analyzed following stimulation with EGF (0-150 ng/mL) for 30 min (A) or EGF treatment for 0 to 120 min at 100 ng/mL (B). C and D, human BMBC cells (231P and 231BR3) were not treated (control) or treated with EGF (100 ng/mL for 30 min following serum starvation for 16 h). Next, immunofluorescent analyses were done using confocal microscopy as described in Materials and Methods. Rabbit polyclonal HPSE antibody (1453) and mouse monoclonal antibody to HPSE were used as primary antibodies (C and D, respectively). They were diluted at 1:1,000 followed by secondary antibody Alexa Fluor 488 goat anti-rabbit IgG or anti-mouse IgG (green staining). Immunofluorescent staining was subsequently analyzed by confocal microscopy. Arrows, HPSE presence in the nucleoli of 231BR3 cells. Red fluorescence, PI staining for cell nuclei. Images were taken at an original objective magnification of ×60. Representative images of four independent experiments are shown. E, staining specificity for anti-HPSE polyclonal and monoclonal antibodies. Anti-rabbit (left) and anti-mouse (right) naive isotypic antibodies (IgG) were used as controls for staining specificity. Cell presence was determined by parallel PI staining. Immunofluorescence and confocal microscopy were subsequently done as per above. Images were taken at an original objective magnification of ×60. F, quantitation of HPSE nucleolar staining in 231P and 231BR3 cells treated or not treated with EGF (100 ng/mL for 30 min at 37°C). Percentage of nucleolar HPSE staining was calculated from the number of nucleoli positive for HPSE staining compared with total nucleolar number obtained by fibrillarin staining. Bars, measurements of 10 fields for each treatment condition and from three independent experiments. **, P < 0.001.
FIGURE 3. EGF-induced HPSE nucleolar localization induces Topo I activity in 231BR3 cells. A, cell fractionation was done as described in Materials and Methods. The fractions were subjected to Western blotting applying anti-HPSE antibody (1453). Purity of fractions was verified using nucleoli-specific (anti-fibrillarin), nuclear-specific (anti-SC35), and cell membrane–specific (anti-integrin β1) antibodies. S, serum-free only (control); E, EGF treatment. B, a representative agarose gel showing EGF-induced Topo I activity. Nucleolar Topo I converts supercoiled DNA (Sc, provided by Topo I assay kit, TopoGen, Inc.) into a relaxed DNA form. Samples were treated with EGF (100 ng/mL) as indicated. Nucleoli were derived from (A) above. C, Topo I activity assays. Briefly, nucleoli were isolated from 231P and 231BR3 cells following treatment with or without EGF (100 ng/mL) for 30 min as per A. Topo I activity was determined by its ability to produce the relaxed DNA form, and was calculated as a ratio of relaxed/supercoiled DNA normalized against nontreated 231P. Three independent experiments were done. **, P < 0.001.

FIGURE 4. Effects of recombinant human HPSE on Topo I activity in 231BR3 nucleoli. Representative experiments in which (A) recombinant human active HPSE (rHAHPSE) and (B) inactive HPSE (rHHPSE) were added to nucleoli isolated from 231BR3 cells (no EGF treatment) at indicated concentrations (0, 10, and 100 ng/mL), following incubation for 30 min at 37°C with supercoiled DNA (100 ng) provided by the TopoGen kit. Topo I activity was examined by 1% agarose gel electrophoresis with ethidium bromide, was visualized using the UV transilluminator, and was determined by DNA status. Relaxed DNA (Rel) indicates Topo I activity, whereas supercoiled DNA (Sc) indicates a lack of Topo I activity. Rel and Sc DNA were used as positive/negative marker controls, respectively. Lower panels were prepared upon densitometric analyses from three independent experiments. Columns, mean measurements from three independent experiments; bars, SD. *, P < 0.01; **, P < 0.001.
detected with avidin-peroxidase, and the absorbance is measured at 450 nm.

HPSE activity was determined as the inverse of decrease in absorbance, per kit instructions (20). BMBC cells (5.0 × 10⁶) were harvested for each cell line and lysed using the extraction buffer supplied by the kit. Then, a 2-fold dilution was prepared by mixing (1:1) the extracted sample with the reaction buffer, and the assay was carried out according to the manufacturer’s protocol. Color was developed using the substrate supplied by the kit and plates were read at 450 nm using a microplate reader (EL 808; Biotek Instruments, Inc.). All samples and readings were done in triplicate.

DNA Topo I Activity Assays

Cells were treated with or without EGF (100 ng/mL, 30 min), then nucleoli were isolated as described above. Topo I activity was determined in isolated nucleoli by using a corresponding kit (TopoGen, Inc.), following the manufacturer’s instructions. Briefly, 20 μL of reaction containing nucleoli extract or/and human recombinant HPSE [active (rhaHPSE) or inactive (rhiHPSE)] protein were incubated with supercoiled DNA (100 ng, provided by the kit) in 1× reaction buffer [10 mmol/L Tris-HCl (pH 7.9), 1 mmol/L EDTA, 1.5 mol/L NaCl, 0.1 mmol/L spermidine, 50% glycerol, 0.1% bovine serum albumin] for 30 min at 37°C. Reaction volumes for different conditions were subsequently loaded on 1% agarose gel and run in 1× TAE buffer.
30 min at 37°C and terminated by adding stop loading buffer (1% sarkosyl, 0.025% bromophenol blue, 5% glycerol). Samples (20 μL) were then loaded on a 1% agarose gel and run in 1 × TAE [40 mmol/L Tris-Acetate, 1 mmol/L EDTA (pH 8.3)] buffer. Following staining with ethidium bromide for 20 min and destaining in distilled water, gels were photographed using a UV transilluminator. Relaxation of supercoiled DNA (Topo I activity) was subsequently assessed and data were quantified by densitometric analyses. Results were expressed by the ratio of relaxed to supercoiled DNA and compared with control group.

**Cell Proliferation Assays**

Cells (231BR3) were treated with or without EGF and LS as specific HPSE inhibitor (21). HPSE, Topo I activity, and cell proliferation analyses were subsequently done. Cells were seeded in 96-well plates at a density of 1 × 10⁴ cells per well in complete DMEM/F12 medium containing 10% fetal bovine serum and antibiotics. Following overnight (16 h) incubation at 37°C, cells were washed with serum-free DMEM/F12 and subsequently incubated for 24 h with serum-free DMEM/F12 containing 0.1% bovine serum albumin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite in the absence or presence of indicated concentrations of LS and/or EGF. Cell proliferation was monitored using a MTS-based nonradioactive proliferation assay (CellTiter 96⁸Aqueous One Solution kit; Promega). Cell proliferation was monitored by determining the absorbance values (490 nm) using a microplate reader, as per the manufacturer’s instructions.

**Statistical Analyses**

Results were quantified and mean, SD, and the number of independent experiments done are presented in all data analyzed. Statistical significance was determined by Student's t or the χ², tests and P values calculated. A P value of <0.05 was considered statistically significant.

**Results**

**HPSE Is Expressed in Human BMBC Cells Possessing a Differential HER2 Content**

To determine the roles of HPSE in HER2/EGFR mechanisms of human BMBC, HPSE presence and HER2 levels were examined by Western blotting in human BMBC cell lines (MDA-231-BR1, MDA-231-BR2, and MDA-231-BR3) and results were compared with 231P counterparts. HPSE expression was investigated using a rabbit anti-HPSE antibody (1453), which detects both the active (50 kDa) and inactive (65 kDa) forms of the

**FIGURE 6.** Double knockdown of HER2/EGFR abolishes HPSE nucleolar localization in 231BR3 cells. Specific siRNA for (A) EGFR, (B) HER-2, or (C) both (HER2 and EGFR), were transfected into 231BR3 cells using FuGene HD transfection reagents. After 48 h, cell lysates were prepared and total proteins (50 μg) were used for the detection of HER2, EGFR, and HPSE by Western blot analyses. D, effect of HER2/EGFR knockdown on HPSE nucleolar localization. 231BR3 cells were first knocked down by indicated siRNAs for 24 h, followed by treatment by EGF (100 ng/mL for 30 min at 37°C). Immunofluorescent assays were then done using anti-HPSE antibody (1453, green fluorescence) and anti-fibrillarin mouse antibody (blue fluorescence). Results were analyzed by confocal microscopy. Images were taken at an original objective magnification of ×60. Arrows, HPSE presence in the nucleoli. Red fluorescence, PI staining for cell nuclei. Representative images of four independent experiments.
enzyme. HPSE content correlated with endogenous HER2 levels and brain metastatic propensities of BMBC cell lines considered (Fig. 1A), particularly in its active form (231BR3 > 231BR2 > 231BR1 > 231P). Next, we examined HPSE enzymatic activity among these cell lines, and detected an increase of HPSE activity consistent with its protein content, with 231BR3 possessing the highest enzymatic activity levels (Fig. 1B). Considering that all cells in this model system express elevated EGFR levels (2, 11, 12), we examined whether EGF modulates HPSE protein expression through HER2 and EGFR phosphorylation in 231P-selected BMBC variants, treated (E) or not treated with EGF in serum-free conditions (S; ref. 11). The expression and phosphorylation of HER2 were higher in 231BR3 cells, when compared with 231P cells, and were accompanied by a corresponding increased HPSE content (Fig. 1C). This was obtained using optimized conditions for EGF treatment (100 ng/mL for 30 minutes at 37°C) as determined by dose- and time-dependent studies (see Fig. 2A and B). Based on these data, we subsequently investigated the EGF treatment modulation of HPSE activity and found that HPSE was increased ~2-fold following EGF treatment of 231BR3 cells (**, *P < 0.001) compared with control (no EGF treatment). Of note, HPSE activity was 2.5-fold higher in 231BR3 compared with 231P cells following EGF treatment (**, *P < 0.001; Fig. 1D). This is relevant to indicate both the involvement of HPSE in BMBC modalities and the regulation of its enzymatic activity by EGF.

**EGF Induces HPSE Nucleolar Localization in High HER2-Expressing 231BR3 Cells**

Considering that HPSE possesses prometastatic functions promoting an aggressive phenotype, and is upregulated by EGF in human BMBC, we sought to investigate EGF-induced HPSE subcellular localization by confocal microscopy, notably to nucleoli. By using fibrillarin as a specific nucleolar marker (18) in 231BR3 cell lysates, we first tested a dose- and time-dependent EGF induction of HPSE nucleolar localization and determined optimal conditions by treating cells with EGF for 30 min at 100 ng/mL (Fig. 2A and B). Second, we observed that HPSE was specifically detected in the nucleoli of 231BR3 cells by using both mouse

![FIGURE 7. Inhibition of HPSE reduces EGF/HPSE-induced Topo I activity and inhibits cell proliferation. 231BR3 cells were treated with EGF (100 ng/mL for 24 h at 37°C) alone or combined with different concentrations of LS or LS alone as indicated in the figure. Treated cells were then divided into three groups and were analyzed for HPSE activity (A), Topo I activity (B), and cell proliferation (C) as described in Materials and Methods. Columns, mean measurements from four independent experiments; bars, SD. *, P < 0.01.]
nucleolar staining in 231BR3 was cellular fractions (14), and is inhibited by HS (15). First, we isolated different expressed in a variety of tumors, including brain neoplasms enriched in nucleoli (13). Of relevance, Topo I is abnormally induced nucleolar HPSE localization, we investigated pos-

Topo I Is a Target of EGF-Induced Nucleolar HPSE

To determine the functional consequences of EGF-induced nucleolar HPSE localization, we investigated possible HPSE targets in nucleoli of BMBC (231BR3) cells. We focused on DNA Topo I because it is an essential enzyme involved in gene transcription and is highly enriched in nucleoli (13). Of relevance, Topo I is abnormally expressed in a variety of tumors, including brain neoplasms (14), and is inhibited by HS (15). First, we isolated different cellular fractions—cytoplasmic, nucleolar, and nucleolar—from 231P and 231BR3 in absence or following EGF treatment. Purity of these fractions was confirmed by Western blotting analyses using corresponding specific markers: integrin β1 (membrane/cytoplasm), SC-35 (nuclear), and fibrillarin (nucleolar) expression. Importantly, the active form of HPSE was detected only in nucleoli preparations of 231BR3 cells following EGF treatment (Fig. 3A). Second, we studied EGF-induced Topo I activity (relaxed form of DNA) and found that it reached a peak at the same EGF conditions (100 ng/mL for 30 minutes) used to detect HPSE nucleolar localization (Fig. 3B). Topo I activity was significantly enhanced (∼3-fold) in 231BR3 cells compared with 231P cells following EGF treatment (Fig. 3C).

To determine the effects of HPSE on Topo I activity in nucleolar fraction, we examined whether Topo I activity was regulated by adding recombinant human HPSE to isolated 231BR3 nucleoli, either as an active (rhaHPSE) or inactive (rhiHPSE) form (3, 8, 9). Results showed that Topo I activity was significantly (∼5-fold) enhanced by adding rhaHPSE (100 ng/assay; Fig. 4A). Conversely, the inactive (not having an endoglycosidase activity, however possessing biological functions; 3, 8, 9) form of HPSE did not significantly alter Topo I activity (Fig. 4B). Next, we used siRNA approaches to knockdown HPSE to show a modulation of Topo I activity. First, a specific siHPSE (SMARTsiRNApool consisting of four nonoverlapping siRNAs) inhibited HPSE expression (Fig. 5A). Second, by applying siHPSE to 231BR3 nucleolar fractions followed by EGF treatment (100 ng/mL for 30 minutes), Topo I activity was significantly inhibited in a dose-dependent manner (Fig. 5B and C). These findings support the notion that Topo I activity is regulated by active HPSE being translocated to nucleoli after HER2/EGFR stimulation by EGF.

Table 1. Nuclear localization of HPSE in BMBL is associated with HER2 overexpression

<table>
<thead>
<tr>
<th>HER2 classification</th>
<th>HPSE nucleolar staining*</th>
<th>Cells with HPSE/Topo I overlay†</th>
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<tr>
<td>Overexpressed</td>
<td>9/11 (81.8%)</td>
<td>72%</td>
</tr>
<tr>
<td>Negative</td>
<td>3/17 (17.6%)</td>
<td>5%</td>
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</tbody>
</table>

NOTE: Human breast cancer specimens were analyzed as described in Materials and Methods.

* Nucleolar staining was determined by fibrillarin staining. Samples percentage represents HPSE nucleolar staining/total number of samples assayed. Nucleolar staining was considered positive only when HPSE/Fibrillarin staining co-localized in >60% of the total cells. Eight to 10 fields were counted for each experiment and were averaged. Overlay of HPSE/Fibrillarin percentage was determined by comparision with Fibrillarin staining only.

† Eight to 10 fields were counted for each slide and averaged. Overlay of HPSE/Topo I percentage was determined by comparison with Topo I staining only.

Table 1. Nuclear localization of HPSE in BMBL is associated with HER2 overexpression

Monoclonal and rabbit polyclonal anti-HPSE antibodies and following EGF exposure (Fig. 2C and D). Normal rabbit and mouse isotypic (IgG) antibodies were also used as staining controls for signal specificity (Fig. 2E). Further, HPSE nucleolar localization/staining positivity in different conditions was quantified. EGF-induced HPSE-positive nucleolar staining in 231BR3 was ∼64% higher compared with 231P cells (**, P < 0.001), and positive staining was increased to 76% in 231BR3 cells following EGF treatment (**, P < 0.001) compared with control (no EGF treatment; Fig. 2F). These findings further support a causal relationship between EGF, HER2/EGFR activation, and EGF-induced regulation of HPSE presence in subcellular compartments.

HPSE Knockdown Reduces Topo I Activity and Cell Proliferation

To show whether HPSE-dependent Topo I activity modulates cell proliferation, we applied siHPSE to 231BR3 cells. When HPSE was siRNA knockdown, Topo I activity was reduced ∼2.2-fold (EGF + siHPSE) and cell proliferation was reduced by ∼40%, compared to EGF treatment alone (Fig. 5D).

To confirm whether HPSE nucleolar localization is HER2/EGFR dependent, we used specifically designed siRNAs for HER2 (siHER2) and EGFR (siEGFR). The inhibitory function of siEGFR and siHER2 were examined by Western blotting (Fig. 6A and B). A double knockdown of HER2/EGFR diminished HPSE expression, especially its active form (50 kDa), which was completely inhibited by this treatment (Fig. 6C). We also visualized HER2/EGFR knockdown effects on HPSE nucleolar localization by confocal microscopy. Using siHER2 or siEGFR alone, HPSE nucleolar localization was only partially inhibited. Conversely, their concurrent use completely abolished nucleolar HPSE presence, similar to siHPSE-transfected 231BR3 cells that were used as control for specificity (Fig. 6D).

Finally, we showed the effects of EGF/HPSE-enhanced Topo I activity on tumor cell proliferation. As shown in Fig. 7, when 231BR3 cells were incubated (24 hours) with a specific inhibitor to HPSE, LS (21), in the presence or absence of EGF, a significant change in cell proliferation was detected in these cells. Cell proliferation was greatly reduced in the presence of LS, and by a concentration-dependent effect (Fig. 7). Data support the notion that HPSE/Topo I mechanism plays an important role in...
EGF-induced in vitro BMBC cell proliferation, which may affect their metastatic behavior in vivo.

**HPSE Correlates with the HER2 Expression Status in BMBC Clinical Specimens**

To investigate whether HPSE nucleolar localization is a relevant property in vivo, we analyzed the localization of HPSE in human BMBC tissues that were either positive (HER2 overexpression) or negative for HER2 content. Patient BMBC samples were collected and sections were stained with H&E as described in Materials and Methods (11). A significant correlation was detected between HER2 positivity and HPSE nucleolar localization (Table 1; Fig. 8). Expression and subcellular localization of HPSE and Topo I were also examined: their distinct colocalization in nucleoli was found at significantly higher cell proportion in HER2-positive (overexpressing) compared with HER2-negative cases (Table 1). HPSE nucleolar staining was detected, in 81.8% and 17.6%, of HER2-overexpressing/HER2-negative groups, respectively. Furthermore, HPSE correlated with Topo I expression and localization in these cases, and overlay of cell number with HPSE/Topo I was determined to be 72% and 5%, respectively (Table 1).

**Discussion**

In this report, we provide first-time evidence showing that (a) HPSE is expressed and functional in human BMBC cells and correlated with HER2 content; (b) EGF induces HPSE localization in the nucleoli of brain metastatic 231BR3 cells; (c) HPSE enhances DNA Topo I activity in the nucleoli and mediates Topo I-dependent BMBC cell proliferation; and (d) nucleolar HPSE is detected in BMBC patient specimens, colocalizes with Topo I, and correlates positively with HER2 overexpression.

It is well-known that HPSE, the only functional mammalian HS endoglycosidase (endo-β-D-glucuronidase), is fundamentally associated with cancer metastasis, a notion...
that has been validated in several in vitro and in vivo settings (3-8). However, HPSE function, particularly in relation to the neoplastic nuclear microenvironment and gene transcription regulation, has not been fully elucidated. Similarly, although HER2 overexpression correlates with BMBC onset (11), precise mechanisms of HER2 action and target molecules are not known. We have discovered that EGF, the natural ligand for HER2/EGFR heterodimer, modulates HPSE expression and activity, and promotes its nucleolar translocation as active enzyme. As a consequence, HPSE function affects Topo I activity, which enhances BMBC cell proliferation.

The subcellular localization of HPSE may be indicative of its different functions. For example, several publications have shown that HPSE can localize to nucleus in which it is active (capable of degrading nuclear HS; ref. 22) and affects cell differentiation (23-25). Moreover, a recent study indicated that active (58-kDa heterodimer) and inactive (65-kDa form) HPSE proteins augment EGFR phosphorylation and associate with tumor progression in head and neck cancers (9). The authors further showed that enhanced EGFR phosphorylation is dependent on HPSE localization. However, the roles and mechanisms of HPSE in the nucleoli, and consequent biological outcomes, have not been investigated. Here, we report another layer of complexity in the HPSE biology: the contrasting effects of nuclear versus nucleolar HPSE on cell proliferation. We propose a novel pathway (EGF-HER2/EGFR-HPSE-Toxo I) in which HPSE is under EGF regulation. The activation of this pathway will lead to the production of active (capable of degrading nuclear HS) HPSE. The HPSE enzyme then translocates to the nucleolus, and Topo I activity is enhanced, which facilitates cell proliferation.

DNA Topo I is an enzyme present in higher eukaryotes and is essential in the regulation of DNA replication by forming covalent complexes that are required for DNA relaxation (Topo I activity) during cell replication and transcription (26). It has been shown that Topo I interacts with many genes that affect cell cycle regulation and proliferation (26-28). Importantly, Topo I is highly present in the nucleoli of mammalian cells (13, 26) and known to bind heparin and HS (15).

We have identified Topo I as a target of nucleolar HPSE. Our results show that HER2/EGFR, HPSE, and Topo I are coinvolved in BMBC proliferative mechanisms, each one playing a specific role. This pathway suggests that the precise subcellular localization of HPSE, e.g., nuclear versus nucleolar, can regulate opposite cellular fates, promoting either differentiation (nuclear) or proliferation (nucleoli). Finally, we have found that the nucleolar localization of HPSE observed in human BMBC cell lines was confirmed in human BMBC tissues overexpressing HER2. The most reasonable explanation for this observation is that cancer patients often experience inflammation and tumor microenvironmental changes that result in the release of growth factors, such as EGF, performing functions similar to ones observed in vitro (29).

In conclusion, the results presented in this study showed that (a) EGF induces nucleolar HPSE localization through HER2/EGFR modalities; (b) nucleolar HPSE enhances Topo I activity and modulates Topo I–dependent cell proliferation; and (c) nucleolar HPSE may be implicated in tumor growth and metastasis in HER2-overexpressing BMBC cases. These findings will provide impetus to investigate HPSE modalities further for clinical intervention and new anti-BMBC therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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