












HS2ST1-dependent signaling pathways determine breast cancer cell viability, matrix interactions, and invasive behavior

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Abstract

Heparan sulfate proteoglycans (HSPGs) act as signaling co-receptors by interaction of their sulfated glycosaminoglycan chains with numerous signaling molecules. In breast cancer, the function of heparan sulfate 2-O-sulfotransferase (*HS2ST1*), the enzyme mediating 2-O-sulfation of HS, is largely unknown. Hence, a comparative study on the functional consequences of *HS2ST1* overexpression and siRNA knockdown was performed in the breast cancer cell lines MCF-7 and MDA-MB-231. *HS2ST1* overexpression inhibited Matrigel invasion, while its knockdown reversed the phenotype. Likewise, cell motility and adhesion to fibronectin and laminin were affected by altered *HS2ST1* expression. Phosphokinase array screening revealed a general decrease in signaling via multiple pathways. Fluorescent ligand binding studies revealed altered binding of fibroblast growth factor 2 (FGF-2) to *HS2ST1*-expressing cells compared with control cells. *HS2ST1*-overexpressing cells showed reduced MAPK signaling responses to FGF-2, and altered expression of epidermal growth factor receptor (EGFR), E-cadherin, Wnt-7a, and Tcf4. The increased viability of *HS2ST1*-depleted cells was reduced to control levels by pharmacological MAPK pathway inhibition.

*Juliana M. Motta and Martin Götte are shared senior authors of this study.

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Moreover, MAPK inhibitors generated a phenocopy of the *HS2ST1*-dependent delay in scratch wound repair. In conclusion, *HS2ST1* modulation of breast cancer cell invasiveness is a compound effect of altered E-cadherin and EGFR expression, leading to altered signaling via MAPK and additional pathways.

KEYWORDS

2-O-sulfotransferase, breast cancer, heparan sulfate, MAPK signaling pathway, proteoglycan

1 | INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are glycoproteins of cell surfaces and the extracellular matrix (ECM) carrying unbranched polysaccharide chains called glycosaminoglycans (GAGs).¹⁻³ HS GAGs contain repetitive disaccharide units comprised of alternating uronic acids and *N*-acetyl-glucosamine that are extensively modified by sulfation.¹⁻³ HSPGs have crucial regulatory roles in normal physiology and pathophysiological conditions, like inflammation, immunomodulation, and tumor onset and progression.³⁻⁵ Cell surface HSPGs immobilize and regulate ECM turnover, providing cytokines, growth factors, and other signaling molecules to receptors on cancer cells to support growth, survival, motility, and angiogenesis.^{1,2,6} They also cooperate with integrins and adhesion receptors to facilitate cell-ECM attachment, cell-cell interactions, and cell motility.^{2,7}

HS biosynthesis begins in the Golgi apparatus with the attachment of a linker tetrasaccharide to a core protein serine residue. A backbone of repeated *N*-acetyl glucosamine (GlcNAc) linked to α -glucuronic acid (GlcA) is synthesized on this linker and extended by the glycosyltransferases-EXT1 and EXT2.^{2,8} Subsequent modification includes *N*-deacetylation and *N*-sulfation of some GlcNAc units to *N*-sulfated glucosamine (GlcNS), catalyzed by *N*-deacetylase sulfotransferases (NDSTs), epimerization of some GlcA to iduronate (IdoA) catalyzed by C5-epimerase, followed by 2-*O*-sulfation of uronate residues catalyzed by HS 2-*O* sulfotransferase (*HS2ST1*, 2-OST).^{1,2} 6-*O*-Sulfation of GlcNAc/GlcNS is catalyzed by HS 6-*O* sulfotransferases, whereas rare 3-*O*-sulfation modifications of the GlcNAc or GlcNS units are mediated by 3-*O*-sulfotransferases.^{1,9}

Cells normally try to maintain the net overall sulfation profile constant.¹⁰ A change in sulfation status at one ring position can be compensated by sulfation at other positions by tightly regulated activities of HS-modifying enzymes in the postulated GAGosome.¹⁰⁻¹³ Key structural determinants of HS interaction with cytokines are the degree, pattern and clustering of sulfated disaccharides along the HS chain,^{9,14-17} which influence the binding ability of HSPG co-receptors to the ligands.

The present study focuses on the role of the HS biosynthetic enzyme *HS2ST1* in breast cancer progression.^{12,18,19} Previous studies in model organisms have demonstrated a role for 2-*O*-sulfation in cell migration and differentiation through unknown pathways.^{20,21} Although HSPGs play an important role in malignancies,²²⁻²⁴ very little is known about the specific functional role of *HS2ST1* in cancer pathogenesis. We show that *HS2ST1* upregulation in breast cancer

cell lines reduces their migratory and invasive behavior due to a decrease in epidermal growth factor receptor (EGFR) and E-cadherin expression and overall phosphokinase signaling. Phenotypic effects are associated with altered binding of growth factors to 2-*O* sulfated HS, and depend on MAPK signaling. These results demonstrate for the first time that increased *HS2ST1* expression controls the invasiveness of breast cancer cells.

2 | MATERIALS AND METHODS

2.1 | Materials

Medium, fetal calf serum (FCS) and tissue culture supplies were from Gibco BRL. Unless stated otherwise, all chemicals were from Sigma.

2.2 | Cell culture

MCF-7 and MDA-MB-231 breast cancer cells were purchased from ATCC/LGC Promochem. Cells were stably transfected as described²⁵ with a pcDNA3.1 control plasmid (Invitrogen) or a plasmid allowing for expression of the open reading frame (1104 bp) of human *HS2ST1* (NCBI Reference Sequence: NM_012262) in the vector pReceiver-M02 under the control of the cytomegalovirus (CMV) promoter (RZPD/ImaGenes). MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS, 1% glutamine, 1% penicillin/streptomycin and 800 μ g/mL G418 in a humidified atmosphere of 7.5% CO₂ in air at 37°C. MCF-7 cells were cultured in RPMI-1640 medium containing 10% FCS, 1% glutamine, 1% penicillin/streptomycin and 800 μ g/mL G418 in a humidified atmosphere of 5% CO₂ in air at 37°C. In some experiments, 10 μ mol/L U0126 (Cell Signaling Technologies) was used to inhibit the MAPK pathway.

2.3 | *HS2ST1*siRNA knockdown

siRNA knockdown was performed using Dharmafect (Dharmacon) and 20 nmol/L validated silencer select siRNA #18569, targeting exon 4 of *HS2ST1*, siRNA #18570, targeting exon 2 of *HS2ST1*, and negative control siRNA #1 (Ambion) according to the manufacturer's instructions. Cellular phenotypes of siRNA-treated cells were

analyzed after 48 h (PCR) or 72 h (protein-based assays, functional assays), respectively.

2.4 | Disaccharide analysis of glycosaminoglycans

GAG isolation was performed from cell pellets as described earlier²⁶ except for elution of DEAE columns with 2 mol/L NaCl followed by desalting on PD MiniTrap™ G-25 columns (GE Healthcare). GAGs were digested with 50 mU of chondroitinase ABC (Seikagaku) and 10% of the total sample directly analyzed by reverse-phase ion-pairing high-performance chromatography (RPIP-HPLC). The remaining 90% were reapplied to a DEAE column and eluted to remove CS-disaccharides. The eluted HS pool was split into 2 equal aliquots and one incubated without, the other with, a mixture of 0.4 mU each of heparin lyases I, II and III (IBEX). Disaccharide separation was performed by RPIP-HPLC as described.²⁶ Peaks were identified by co-elution with standard CS and HS disaccharides.

2.5 | Cell viability assay

Cell viability was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay essentially as previously described.²⁷ Here, 5000 cells were seeded in quadruplets into 96-well plates and cultured for 24, 48, or 72 h, respectively. Afterwards, 4 h of incubation in the presence of 5 mg/mL MTT followed. Cells were lysed by addition of an equal volume of 10% sodium dodecyl sulfate (SDS) in 50% *N,N*-dimethylformamide, pH 4.7, for 16 h, and optical density measurement at 595 nm was performed using a microplate reader. Cell viability data are shown as percentage viability of vector/control siRNA-treated cells.

2.6 | Apoptosis assay

Apoptotic cells were identified by flow cytometry using the Annexin V test kit (Becton Dickinson), based on their property of binding fluorescently labeled annexin V as a result of apoptosis-related membrane inversions. Cells were counterstained with propidium iodide to identify late apoptotic/necrotic cells. Fluorescence emission was measured at 527 and 675 nm on a flow cytometer (CyFlow Space, Partec).

2.7 | Invasion assay

BioCoat Matrigel Invasion Chamber (BD Biosciences) assays were performed exactly as described previously.^{25,27} Briefly, 25 000 cells were seeded into invasion filters (BD Biosciences) in a 24-well plate format and incubated for another 24 h. Invasion was induced by addition of serum-free medium to the upper chamber and of 10% FCS-containing medium as chemoattractant to the bottom chamber

of the invasion chamber for another 24 h (MDA-MB-231) or 48 h (MCF-7), respectively, resulting in transmigration of the cells through the basement-membrane-like extracellular matrix. Cells in the upper chamber were removed with a cotton swab, and cells on the lower surface were fixed and stained with Diff-Quik dye (Medion). Excised and mounted filter membranes were photographed using a Zeiss Axiocvert microscope (Zeiss) at $\times 100$ magnification. Relative invasiveness was expressed as percentage of the HS2ST1-overexpressing or siRNA-treated cells compared with vector/control siRNA-treated cells ($n > 3$).

2.8 | Scratch motility assay

Scratch motility assays were performed as previously described.²⁷ Confluent cell monolayers were wounded by scraping once horizontally and vertically with a 100 μ L pipette tip. Closing of the scratch wound was monitored by Nomarski contrast light microscopy and documented with a Zeiss Axiophot camera (Zeiss) immediately and 6 h after wounding. Zeiss Axiovision software (Zeiss) was used to calculate the cell-free wounded area. Data are shown as percentage of the cell-free area of HS2ST1-manipulated cells compared with controls.

2.9 | Migration assay and digital holographic microscopy

An inverted microscope (iMIC, Till Photonics) with an attached digital holographic microscopy (DHM) module²⁸ and an incubator (Solent Scientific Ltd.) for stabilized temperature were used for bright-field imaging and quantitative digital holographic phase-contrast imaging of control and HS2ST1-overexpressing MDA-MB-231 cells seeded on laminin-coated Petri dishes. The coherent light source for the recording of digital holograms was a frequency-doubled neodymium-doped yttrium aluminum garnet (Nd:YAG) laser (Compass 315 M-100, Coherent, $\lambda = 532$ nm). Experiments were performed in μ -dishes (ibidi GmbH) seeded with 3×10^4 cells. Digital holograms of single cells were recorded continuously every 3 min. Digitally captured hologram reconstruction was performed by spatial phase shifting reconstruction.²⁹ For DHM, the cells were cultured in HEPES-buffered DMEM. Three independent single cell analyses were performed and representative measurements are shown in the results. Migratory behavior of cells was analyzed by automated cell tracking.³⁰

2.10 | Adhesion assay

Here, 96-well plates were coated with 10 μ g/mL fibronectin or 50 μ g/mL laminin or 10 μ g/mL BSA (negative control) for 16 h at 4°C and washed twice with wash solution (0.1% BSA in DMEM), followed by blocking of nonspecific binding with 0.5% BSA in DMEM at room temperature. Adhesion of 2.5×10^4 cells/well was quantified

using a photometric methylene blue staining based method as previously described.⁷ Cells were released with 2 mmol/L EDTA in PBS, washed twice, and resuspended in blocking solution prior to incubation with the coated wells for 1 h at 37°C. Nonadherent cells were removed by 3 washes with PBS. Following fixation of attached cells with 3.7% NaCl/PBS-buffered formaldehyde for 30 min, cells were stained with 1% methylene blue in 0.01% borate buffer (pH 8.5) for 30 min. Following 4 washes with borate buffer, the cells were lysed in ethanol/0.1 mol/L HCl (1:1), and the released stain was quantified in a Softmax Microplate reader (Molecular Devices) at 620 nm. Data are expressed as percentage of *HS2ST1*-manipulated cell values compared with control cells.

2.11 | Quantitative real-time PCR

Total cellular RNA was isolated using RNA-OLS (OMNI Life Science) and reverse transcribed (Advantage First strand cDNA synthesis kit; Fermentas). qPCR and melting curve analysis were performed using Qiagen QuantiTect SYBR Green PCR kit in a LightCycler. Expression of additional mRNAs was analyzed using TaqMan probes on an ABI PRISM 7300 Sequence Detection System as described previously using the $2^{-\Delta\Delta Ct}$ method after normalization to 18S rRNA.²⁷ The following TaqMan probes were used (see also lifetechnologies.com): 18S rRNA: hs 99999901_s1; EGFR: hs 00193306_m1; HS2ST1: hs_00202138_m1; Wnt 7a: hs 01114990_m1.

2.12 | Western blotting

Western blotting was performed as previously described.⁷ Briefly, cells were lysed in modified RIPA buffer with proteinase inhibitors. 50 µg of protein per lane were separated on 10% gels, and electrotransferred to Hybond nitrocellulose membranes (Amersham, Pharmacia Biotech). For antibody incubations, the membranes were blocked with 5% skimmed milk in 0.1% TBS-Tween (TBST) for 1 h at room temperature, followed by incubation with the primary antibody overnight at 4°C. Antibodies and dilutions are listed in Table 1. After washing in TBST buffer 3 times, the membranes were incubated for 30 min at room temperature with horseradish peroxidase-conjugated secondary antibodies in blocking buffer. The membranes were washed and treated with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) for 1 min and exposed to Hyperfilm-ECL. For detection of the total forms of phosphorylated proteins, and for detection of the housekeeping control protein tubulin, membranes were stripped with 0.2 mol/L glycine buffer (pH 2.5), washed, reincubated with primary antibody, and subjected to the procedure described above.

2.13 | Phosphokinase array

Human phosphokinase array was performed following manufacturer's instructions (R&D Systems). 300 µg of protein from control and

TABLE 1 Antibodies used in this study

Antibody	Working dilution	Manufacturer
Rabbit polyclonal anti-phospho-Thr202/Tyr204 p44/42 MAPK	1:1000	Cell Signalling
Rabbit polyclonal anti-p44/42 MAPK	1:1000	Cell Signalling
Mouse anti-human E-cadherin, clone 36	1:1000	Becton and Dickinson Biosciences
Rabbit polyclonal anti-EGFR	1:1000	Cell Signalling
Rabbit monoclonal anti-human TCF4	1:1000	Cell Signalling
Rabbit polyclonal anti-HS2ST1	1:1000	Cell Signalling
Rabbit polyclonal anti-phospho-Thr180/Tyr182 p38 MAPK	1:1000	Cell Signalling
Rabbit polyclonal anti-p38 MAPK	1:1000	Cell Signalling
Mouse anti-human α -Tubulin, clone B-5-1-2	1:4000	Sigma-Aldrich
Horseradish peroxidase-conjugated goat-anti-mouse IgG	1:10 000	Merck-Millipore
Horseradish peroxidase-conjugated goat-anti-rabbit IgG	1:2000	Merck-Millipore
Horseradish peroxidase-conjugated rabbit-anti-goat IgG	1:5000	Merck-Millipore

HS2ST1-overexpressing cells was analyzed in a panel of phosphorylation profiles of kinases.

2.14 | Analysis of biotinylated FGF-2 binding by flow cytometry

Binding of basic fibroblast factor (FGF-2) to 4×10^6 EDTA-detached cells/mL was analyzed by flow cytometry employing Fluorokine^R Biotinylated human FGF-2 and avidin-FITC reagent (R&D Systems) according to the manufacturer's instructions.

2.15 | Heparan sulfate purification from cells for Fourier transform infrared (FTIR) analysis

Here, $\sim 2.5 \times 10^7$ cells per sample were washed in PBS, scraped from the flasks, pelleted and lyophilized. The lyophilized cell pellets were separately suspended in 2 mL of 0.1 mol/L sodium acetate buffer (pH 5.5) containing 1% of papain, 5 mmol/L EDTA and 5 mmol/L cysteine and incubated at 60°C overnight. This was then centrifuged at 2000 g for 10 min at room temperature, the supernatant was separated. The clear supernatants from different samples were ethanol precipitated and suspended in 0.1 mol/L NaCl. The solution was applied on a DEAE column equilibrated with sodium phosphate buffer (pH 6.0) containing 0.15 mol/L NaCl. Fractions were eluted with 1.0 mol/L NaCl in the same buffer, desalted with HiTrap™

desalting column, lyophilized, and resuspended in 15 μL 0.03 mol/L acetate buffer (pH 7.0) with 1.0 mU/ μL chondroitin ABC (in 10 μL cABC buffer, pH 8.0) to degrade chondroitin sulfate and hyaluronan. The mixture was incubated at 37°C overnight, lyophilized and the enzymatic reaction was inactivated at 96°C for 2 min before freeze drying. Samples were resuspended in Milli-Q water to load onto the HPLC for separation of HS.

2.16 | Extraction of total GAGs from conditioned medium

Cells underwent starvation in growth medium without serum for 24 h at 37°C with 5% CO_2 in air. Then, 10 mL of conditioned medium (CM) were centrifuged to remove cell debris. CM supernatants were concentrated using a Vivaspin™ column with a 10 000 Da MWCO (GE Healthcare Bio-Sciences AB), and incubated overnight at 37°C with a pronase (Cat. No. P8811-1G, Sigma-Aldrich) to digest all proteins. Proteinase deactivation was performed by addition of NaCl (50 nmol/L) and incubation at 100°C for 1 min. After cooling, centrifugation was performed to pellet digested proteins. GAGs were precipitated from the supernatant by addition of saturated sodium acetate and incubation at 4°C for 3 h. Precipitated GAGs were air-dried and resuspended in sterile distilled water. For each cell line, 3 independent biological replicates were analyzed.

2.17 | FTIR spectroscopy of extracted GAGs

Here, 5 μL of resuspended extracted GAGs (1 $\mu\text{g}/\mu\text{L}$) were deposited in triplicate onto a high-throughput 384-well silicon plate, air-dried, and analyzed with a high-throughput screening HTS-XT extension coupled to a Tensor 27 FTIR spectrometer (Bruker Optics GmbH). The FTIR acquisitions of the samples were performed in transmission mode, in the spectral range 4000–400 cm^{-1} , at a spectral resolution of 4 cm^{-1} with 64 scans. Before each sample measurement, the silicon plate background was recorded and automatically removed from the sample signal. One spectrum was obtained from each well. Acquisition and pre-processing were performed with the OPUS software (Version 6.0, Bruker Optics).

2.18 | Spectral data pre-processing and analysis

Before pre-processing, some spectra were discarded after spectral quality test. For FTIR spectral analysis, a baseline correction was performed and a second derivative was calculated to increase the differences between spectra. A vector normalization was applied for spectral comparison. Then, data were processed with multivariate statistical exploratory techniques like hierarchical cluster analysis (HCA) and principal component analysis (PCA). These clustering methods based on distance calculation and variance analysis respectively, allowed for analysis how spectra group together in an unsupervised

way. These 2 analyses were performed in the 1350–900 cm^{-1} spectral range. The complete workflow for GAGs analysis by FTIR high-throughput spectroscopy has been reported previously.³¹

2.19 | Statistical analysis

Data were expressed as mean \pm SEM or SD, as indicated. Statistical analysis was performed using the Sigma Stat 3.1 software (Systat Software). An unpaired *t* test was used when groups passed the normality test, otherwise, the Mann-Whitney *U* test was used. A two-sided *P*-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Confirmation of stable overexpression and transient siRNA knockdown of *HS2ST1*

To study the functional impact of increased *HS2ST1* expression, the metastatic triple-negative cell line MDA-MB-231 and the less dedifferentiated and less invasive hormone-receptor-positive cell line MCF-7 were stably transfected with a control vector and a plasmid overexpressing *HS2ST1* under the control of a strong CMV promoter, respectively. To ensure that the observed phenotypic end points are due to increased *HS2ST1* expression and not to other molecular mechanisms, a complementary transient siRNA knockdown approach was implemented. In addition, key experiments were confirmed using independent overexpressing clones (see Figure S1). qPCR analysis showed an 8–11-fold increase in mRNA expression of *HS2ST1* (Figure 1A and Figure S1A) and up to 90% decrease in its expression upon siRNA knock down (Figure 1B). The change in *HS2ST1* expression was confirmed at the protein level by western blotting (Figure 1C,D).

3.2 | FTIR discrimination of *HS2ST1*-overexpressing cells based on extracted GAGs

Taking into account the specific spectral signatures obtained from standard GAG molecules and GAG mixtures^{32,33} and from cells based on their capacity for GAG synthesis,³⁴ FTIR measurements were undertaken to characterize extracted GAGs from the CM of *HS2ST1*-overexpressing vs control cells. The mean normalized FTIR spectra obtained of extracted GAGs showed differences between MCF-7 and MDA-MB-231 cell lines in the 1800–900 cm^{-1} spectral range. These differences were more visible in the 1350–800 cm^{-1} spectral range (GAGs absorption region). This region was selected for exploratory chemometrics analysis. Moreover, in the 1350–800 cm^{-1} spectral range, extracted GAGs from control and *HS2ST1*-overexpressing MCF-7 or MDA-MB-231 cells could be easily discriminated. HCA and PCA were performed on second derivative spectra in the 1350–800 cm^{-1} spectral range. HCA results displayed

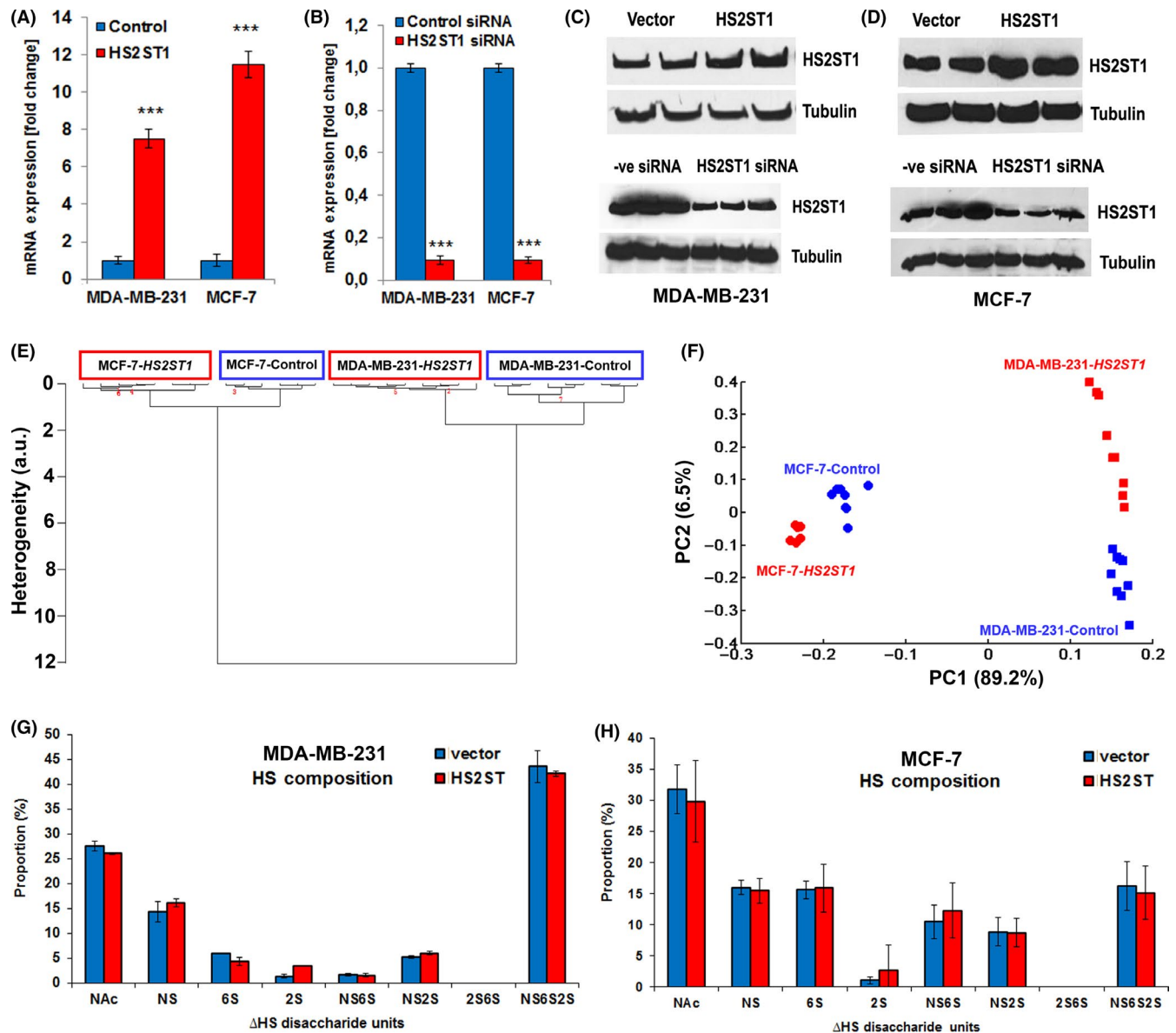


FIGURE 1 Overexpression of *HS2ST1* results in altered FTIR spectra. A, B, qPCR (A, B) and western blotting (C, D) confirm stable transfection (A) and siRNA knockdown (B) of *HS2ST1* in MDA-MB-231 and MCF-7 cells. A, B, N = 9, Error bars = SEM, ****P* < .001. C, D, Upper panels, plasmid-based overexpression; lower panels, siRNA knockdown. E, Hierarchical cluster analysis of FTIR spectra of GAGs from conditioned medium (CM) of control and *HS2ST1*-overexpressing cells. F, Principal component analysis score plot of FTIR spectra of GAGs extracted from CM of control and *HS2ST1*-overexpressing cells. E, F, Analysis was performed on second derivative spectra in the 1350–800 cm^{-1} spectral range. G, H, Biochemical RPIP-HPLC characterization of HS disaccharides of *HS2ST1*-transfected cells. Peaks co-eluting with standard disaccharides are labeled as *N*-acetylglucosamine (NAc), *N*-sulfated glucosamine (NS) and sulfate group at indicated position (S). N = 2 independent analyses

in Figure 1E showed that spectra of GAGs extracted from CM of the same cell type were grouped in the same cluster. They exhibited a low degree of heterogeneity, indicating a good reproducibility, and a low intra-group variability, which was sufficient to distinguish between MCF-7 and MDA-MB-231 CM. Moreover, CM from control and *HS2ST1*-transfected cells could be discriminated. To confirm these observations, a second chemometric method based on PCA analysis was carried out. Figure 1F shows the PCA score plot of the CM of each cell type using the first 2 principal components (PCs) carrying the highest explained variance. The first PC (PC1) carried

89.2% of the total variance and allowed a clear separation between CM from MCF-7 and MDA-MB-231 cell lines. These results also showed that PC2, representing 6.5% of the total variance, allowed the separation of CM between control and *HS2ST1*-overexpressing cells. This is clear for MDA-MB-231 cell types while a combination of PC1 and PC2 seemed to be necessary to delineate between MCF-7 cell types. Disaccharide analysis of *HS2ST1*-overexpressing MDA-MB-231 (Figure 1G) and MCF-7 (Figure 1H) cells also revealed a slight increase of 2-*O*-sulfated HS structures compared with their corresponding vector controls.

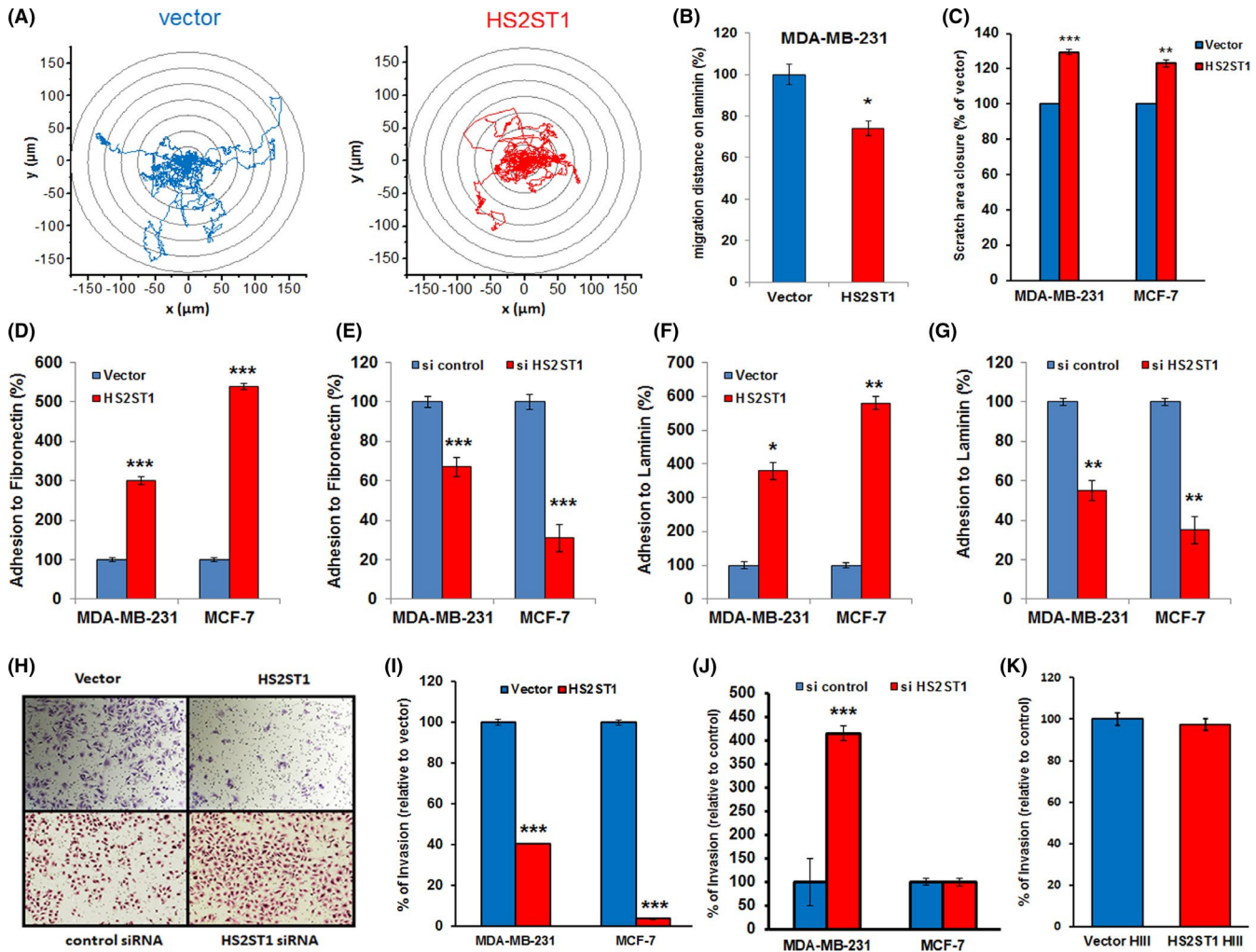


FIGURE 2 *HS2ST1* modulates breast cancer cell adhesion, migration and invasion. A, B, DHM time lapse analysis reveals lower migration of *HS2ST1*-transfected MDA-MB-231 cells ($n = 13$) compared with vector controls ($n = 16$), on laminin coating. A, Migration tracks. B, Quantification revealing a c. 30% decrease in the migration distance. $N = 3$, error bars = SEM. C, Scratch wound closure assay reveals slower migration of *HS2ST1*-transfected MDA-MB-231 and MCF-7 cells compared with controls. *** $P < .001$, ** $P < .01$; $N \geq 9$, error bars = SEM. D-G, Cell adhesion to fibronectin and laminin is increased upon *HS2ST1* overexpression (D, F) and decreased upon *HS2ST1* depletion (E, G). $N = 18$, Error bars = SEM, *** $P < .001$, ** $P < .01$, * $P < .05$. H-K, Impact of *HS2ST1* on invasive behavior of breast cancer cells in Matrigel invasion chamber assays. H, Representative images of Matrigel invasion chamber assays. $t = 24$ h (highly invasive MDA-MB-231), $t = 48$ h (low invasive MCF-7). I, *HS2ST1* overexpression significantly inhibits invasion. J, siRNA knockdown of *HS2ST1* promotes MDA-MB-231 invasiveness, while invasion is not affected in MCF-7 cells. *** $P < .001$, ** $P < .01$, $N \geq 6$, error bars = SEM. K, Vector controls and *HS2ST1*-overexpressing MDA-MB-231 cells displayed no difference in their invasion capacity upon prior HS degradation with 0.5 U/mL heparin lyase III (4 h). $N = 6$, error bars = SEM

3.3 | *HS2ST1* modulates breast cancer cell migration, cell-ECM adhesion, and invasiveness

Migration and cell-ECM adhesion are critical steps during cancer cell invasion that are regulated by HSPGs.^{25,35,36} Hence, we tracked the migratory behavior of *HS2ST1*-overexpressing MDA-MB-231 cells by time lapse microscopy and observed a decrease in their capability to migrate on laminin by ~25% (Figure 2A,B). These results were consistent with data obtained by scratch wound assay, where *HS2ST1*-overexpressing cells displayed a significant delay of 23%–30% in wound closure (Figure 2C and Figure S1B). In vitro adhesion assays revealed that *HS2ST1*-overexpressing MDA-MB-231 cells showed significantly increased adhesion of 3.0-fold to fibronectin

and ~3.8-fold to laminin, while MCF-7 showed a ~5.4-fold increase toward fibronectin compared with ~5.7-fold toward laminin substrate (Figure 2D-G). In contrast, *HS2ST1*-depleted MDA-MB-231 cells displayed a 33% decrease in adhesiveness to fibronectin and ~45% decrease to laminin compared with the controls (Figure 2E,G). The effect was more pronounced in MCF-7 cells, where a decrease of 70% to fibronectin and 66% to laminin was observed (Figure 2E,G). We next investigated the cellular capacity to invade through Matrigel, a basement membrane-like ECM. While invasion decreased in *HS2ST1*-overexpressing MCF-7 cells and in the highly metastatic MDA-MB-231 cells (Figure 2H,I), it dramatically increased upon *HS2ST1* knockdown in MDA-MB-231 (Figure 2H,J). In contrast, siRNA knockdown of *HS2ST1* in MCF-7 cells did not affect invasiveness (Figure 2J). We no

longer observed a difference in the invasive potential of control and *HS2ST1*-overexpressing cells after HS digestion with heparin lyase III, suggesting that indeed the HS and its modification correlate to the observed invasive phenotype (Figure 2K).

3.4 | *HS2ST1* overexpression reduces breast cancer cell viability and signal transduction via multiple pathways

HSPGs play an important role as signaling co-receptors.^{1,2,7,37,38} To test the impact of altered *HS2ST1* expression, cell viability was assessed by MTT assay over a time course of 72 h (Figure 3A). While cell viability was consistently decreased upon *HS2ST1* overexpression in both cell lines at all timepoints after 24, 48 and 72 h, respectively, MDA-MB-231 cells were more strongly affected by *HS2ST1* overexpression compared with MCF-7 cells (Figures 3A and S1C). The *HS2ST1*-dependent decrease in viability was not due to increased apoptosis, as flow cytometric analysis of annexin V binding did not reveal significant differences between *HS2ST1*-overexpressing and control breast cancer cells (Figure S1D). Phosphokinase array screening revealed a

net overall decrease in the phosphorylation status upon overexpression of *HS2ST1* in MDA-MB-231 cells. Phosphorylation of p38 MAPK, Erk 1/2 (p44/42 MAPK), JNK 1/2/3, EGFR, Akt, and p53, along with several other proteins, was decreased in *HS2ST1*-overexpressing compared with control cells, (Figure 3B and Table 2), indicating an important general role of *HS2ST1* in signal transduction. *HS2ST1* overexpression led to decreased activation of the p44/42 and p38 MAPK signaling pathways (Figures 3C,D and S1E,F) in MDA-MB-231 cells. *HS2ST1* knockdown resulted in similar constitutive activation levels of p44/42 MAPK in control and *HS2ST1*-overexpressing MDA-MB-231 cells (Figure 3E). In contrast, siRNA knockdown of *HS2ST1* activated p44/42 MAPK signaling in MCF-7 cells (Figure 3F).

3.5 | Alterations in the migratory and invasive behavior are associated with changes in the expression of the cell-cell adhesion molecule E-cadherin and the transcription factor TCF-4

The prognostically relevant cell-cell adhesion molecule E-cadherin is co-expressed with the HSPG Syndecan-1 in early breast cancer and

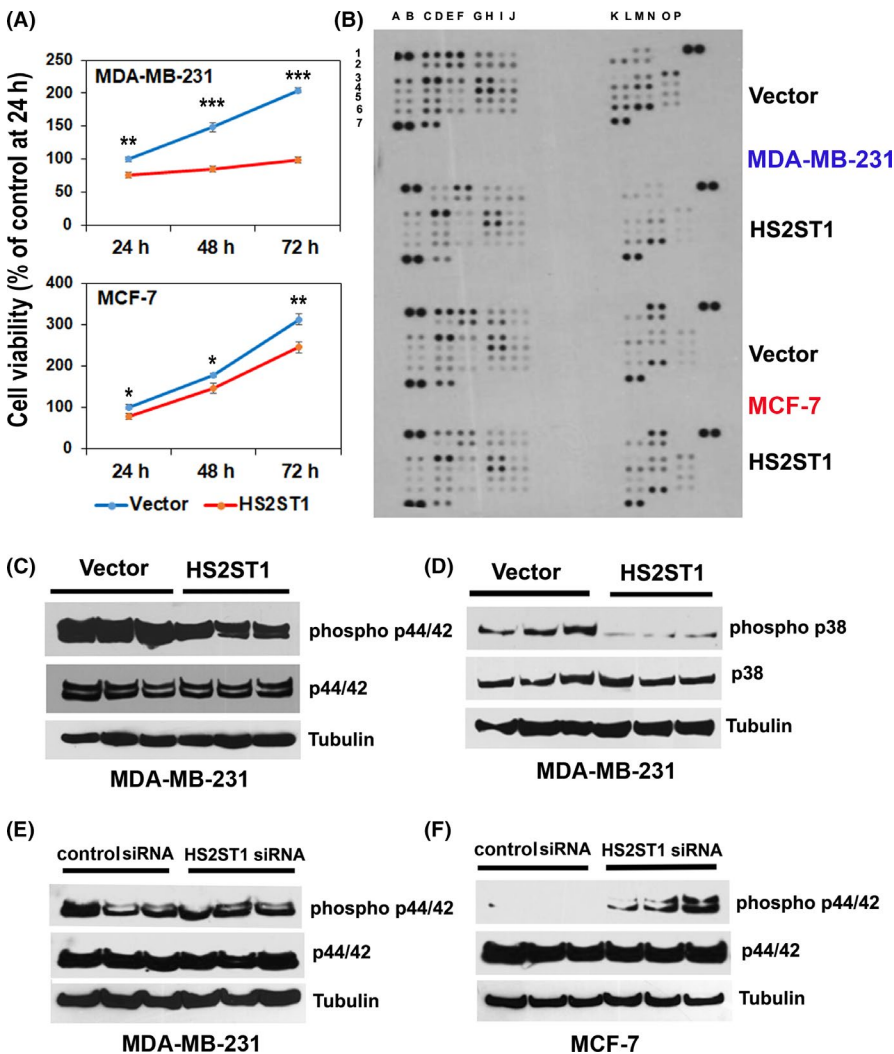


FIGURE 3 *HS2ST1* affects cell viability and modulates multiple signaling pathways. A, *HS2ST1* upregulation significantly reduces cell viability over a time course of 24–72 h (MTT assay), having a stronger impact in MDA-MB-231 cells compared with MCF-7 cells. * $P < .05$, ** $P < .01$, *** $P < .001$, $N = 6$, error bars = SEM. B, Phosphokinase array blot showing an overall decrease in signaling upon *HS2ST1* overexpression compared with controls. C, D, Western blot analysis reveals that constitutive p44/42 (C) and p38 (D) MAPK activation were decreased in *HS2ST1*-overexpressing MDA-MB-231 cells. E, Basal p44/42 MAPK was activated upon *HS2ST1* siRNA knockdown in MDA-MB-231 cells raising the activation levels similar to the controls. F, siRNA knockdown of *HS2ST1* activates basal MAPK in MCF-7 cells compared with controls where the pathway is not active. Tubulin was used as a loading control. $N = 9$

TABLE 2 Relative activation of kinases and signaling receptors in HS2ST1 and control vector-transfected cell lines according to phosphokinase array screening

Number	Protein	Phosphorylation site	HS2ST1-overexpressing cells	
			MDA-MB 231	MCF-7
1 C,D	p 38 alpha	T180/Y182	Down	Down
1 E,F	ERK 1/2	T202/Y204, T185/Y187	Down	Down
1 G,H	JNK 1/2/3	T183/Y185, T221/Y223	Down	Down
2 C,D	EGFR	Y1086	Down	Down
2 E,F	Msk 1/2	S376/S360	Down	Down
2 G,H	AMPK α 1	T183	Down	Down
2 I,J	Akt 1/2/3	S473	Down	No change
2 K,L	Akt 1/2/3	T308	Down	No change
2 M,N	P53	S46	Down	Slightly down
3 A,B	TOR	S2448	Slightly down	No change
3 E,F	HSP27	S78/S82	Down	Slightly down
3 G,H	AMPK α 2	T172	Slightly down	No change
3 I,J	β -Catenin	–	Down	Slightly down
3 K,L	P70 S6 kinase	T389	Down	No change
3 M,N	P53	S15	Down	Slightly up
3 O,P	c-Jun	S63	Down	Up
4 A,B	Src	Y419	Slightly down	Slightly down
4 C,D	Lyn	Y397	Down	No change
4 E,F	Lck	Y394	Down	No change
4 G,H	Stat2	Y689	Slightly down	No change
4 I,H	Stat 5a	Y694	Down	Slightly down
4 K,L	P70 S6 kinase	T421/S424	Down	Slightly up
4 M,N	RSK 1/2/3	S380/S386/S377	Down	Slightly up
4 O,P	eNOS	S1177	Down	No change
5 A,B	Fyn	Y420	Down	No change
5 C,D	Yes	Y426	Down	No change
5 G,H	Stat 6	Y641	Down	Down
5 I,J	Stat 5B	Y699	Down	Down
5 K,L	Stat 3	Y705	Down	Slightly down
5 M,N	P27	T198	No change	Not expressed
5 O,P	PLC- γ 1	Y783	Down	Slightly up
6 A,B	Hck	Y411	Down	Slightly down
6 C,D	Chk-2	T68	Down	No change
6 E,F	FAK	Y397	Slightly down	No change
6 G,H	Stat 5 a/b	Y694/Y699	Down	No change
6 K,L	Stat 3	S727	Down	No change
6 M,N	Wnk 1	T60	Down	No change
6 O,P	Pyk 2	Y402	Down	No change
7 C,D	PRAS 40	T246	Down	Down

regulates the invasive capability of cells.³⁹⁻⁴² While E-cadherin expression was increased in *HS2ST1*-overexpressing cells compared with controls, its expression was downregulated upon *HS2ST1* knockdown in both cell lines (Figure 4A-D). HSPGs modulate the Wnt/ β -catenin signaling pathway, which regulates cell survival, proliferation, and

differentiation.^{43,44} Western blot analysis of the Wnt effector Tcf4 (Tcf7l2) confirmed strong downregulation in *HS2ST1*-overexpressing MDA-MB-231 and MCF-7 cells compared with controls (Figure 4E,F). Pharmacological inhibition of constitutive MAPK signaling in MDA-MB-231 cells expressing a different HS sulfotransferase,

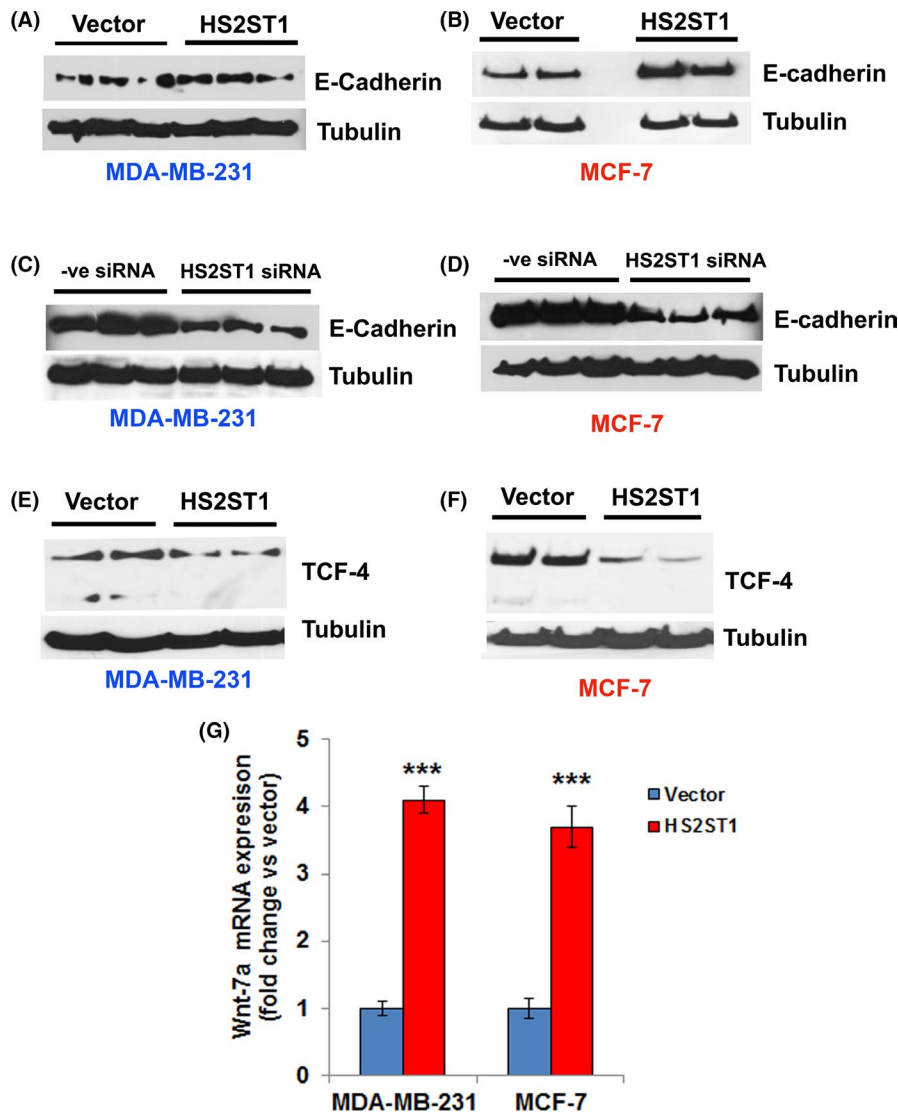


FIGURE 4 *HS2ST1* expression affects the expression of E-cadherin, Tcf4, and Wnt 7a. A, B, Western blot shows increased E-cadherin expression upon *HS2ST1* overexpression. C, D, *HS2ST1* siRNA knockdown decreases E-cadherin expression. E, F, *HS2ST1* overexpression leads to decreased expression of the Wnt-related transcription factor Tcf4. G, qRT-PCR reveals upregulation of *WNT-7a* in *HS2ST1*-overexpressing cells. *** $P < .001$, $N \geq 6$, error bars = SEM

HS2ST2, abolished the increased Tcf4 expression,⁴⁵ suggesting a cross-talk between the 2 pathways. Moreover, *HS2ST1* overexpression led to elevated Wnt 7a mRNA expression (Figures 4G and S1G).

3.6 | *HS2ST1* regulates the expression of epidermal growth factor receptor

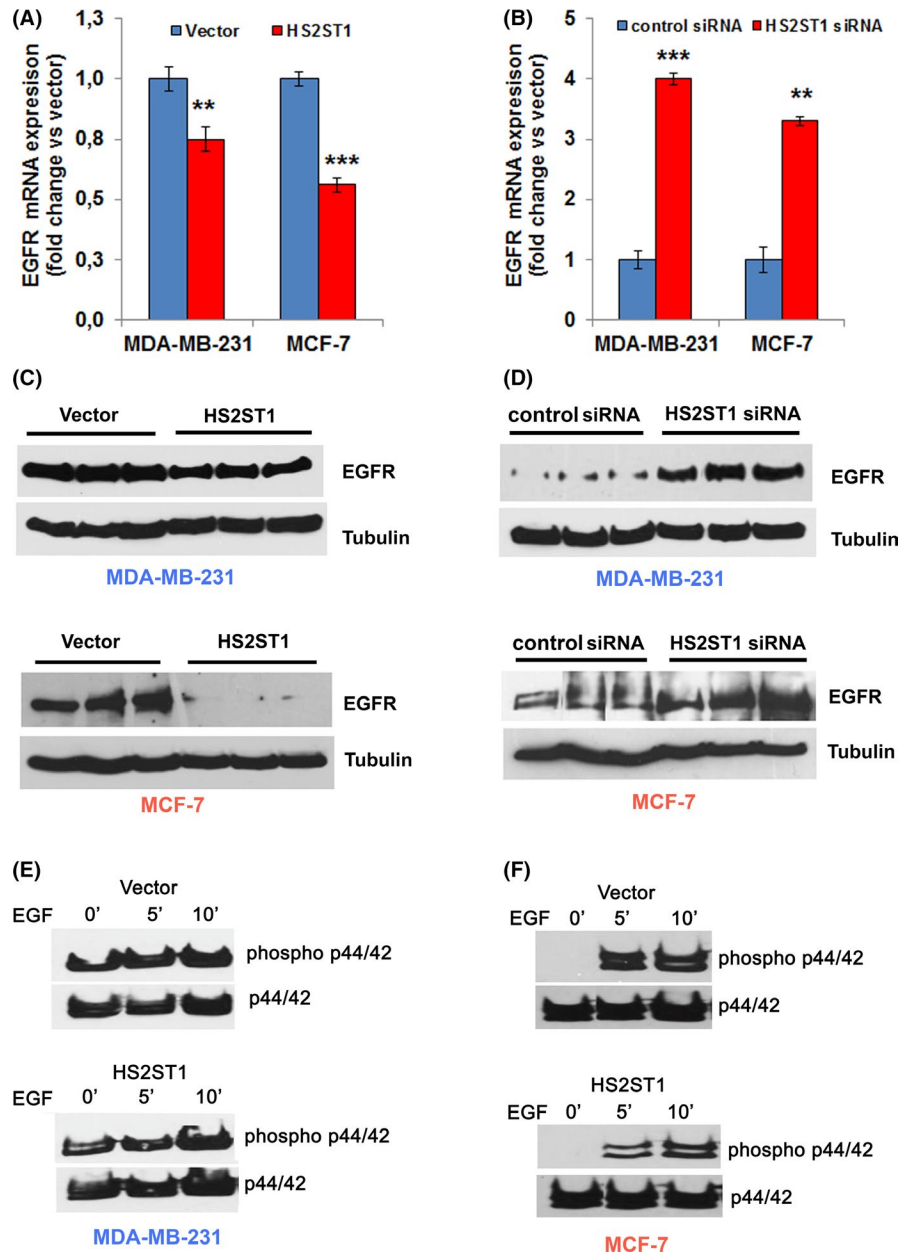
EGFR expression is frequently elevated in breast cancer,⁴⁶ and its activation was affected by *HS2ST1* according to our phosphokinase array screening. qPCR analysis revealed a significant decrease in EGFR mRNA expression levels in *HS2ST1*-overexpressing cells while we observed an increased EGFR mRNA expression upon *HS2ST1* knockdown (Figures 5A,B and S1H). These data were confirmed at the protein level by western blot analysis (Figure 5C,D). We next tested if altered expression of *HS2ST1* affected the activation of MAPK signaling in the presence of specific growth factors. For this purpose, serum-starved *HS2ST1*-overexpressing MDA-MB-231 cells were stimulated with EGF, which resulted in a moderate increase in MAPK activation due to the known constitutive activation status⁴⁵ in this cell line (Figure 5E). In

HS2ST1-overexpressing cells, constitutive and early EGF-induced MAPK activation were reduced compared with controls (Figure 5E). In MCF-7 cells, where the pathway is not constitutively active,⁴⁵ EGF activated p44/42 MAPK after 5 min of stimulation, whereas MAPK activation was reduced in *HS2ST1*-overexpressing cells (Figure 5F).

3.7 | Increased 2-O-sulfation affects binding of FGF-2 to HS and FGF-2-induced p44/42 MAPK activation

In breast and colon cancer, HSPGs modulate basic fibroblast growth factor (FGF-2)-mediated MAPK signaling.^{25,46} FGF-2 signaling is effectively inhibited by 2-O- and N-sulfated HS oligosaccharides competing with cellular HS for binding to FGF-2.⁴⁷⁻⁴⁹ In light of these findings, binding of FGF-2 to control and *HS2ST1*-overexpressing cells was analyzed by flow cytometry by in vitro binding of fluorescently labeled FGF-2. The results were comparable as the *HS2ST1*-overexpressing cells showed in both cell lines a ~20% reduced weaker binding of FGF-2 to HS compared with the controls (Figure 6A). Stimulation of *HS2ST1*-overexpressing

FIGURE 5 *HS2ST1* modulates EGFR expression and growth factor-mediated activation of p44/42 MAPK in breast cancer cells. A, qRT-PCR reveals downregulation of EGFR mRNA expression level in *HS2ST1*-overexpressing breast cancer cells. B, *HS2ST1* siRNA knockdown upregulates EGFR mRNA expression. *** $P < .001$, ** $P < .01$, $N \geq 6$, error bars = SEM. C, Western blot showing decreased EGFR protein expression compared with controls in *HS2ST1*-overexpressing cell lines (upper panels), whereas EGFR expression is increased upon *HS2ST1* siRNA knockdown (D). E, F, *HS2ST1*-overexpressing MCF-7 cells show a decreased response to EGF stimulation. Serum-starved vector and *HS2ST1*-transfected cells were stimulated with 100 ng/mL EGF for 0, 5, and 10 min. Here, 25 μ g of cell lysate/lane were analyzed by western blotting for phospho-p44/42 MAPK, p44/42 MAPK, and the loading control α -tubulin. Compared to controls, EGF treatment slightly decreased p44/42 MAPK activation as a readout of EGF-induced signaling in *HS2ST1*-overexpressing MCF-7 cells. The signaling response was attenuated to a lower extent in *HS2ST1*-overexpressing MDA-MB-231 cells



MDA-MB-231 cells with FGF-2 resulted in a weaker activation of the p44/42 MAPK signaling pathway compared with control cells (Figure 6B). In MCF-7 cells, FGF-2 activated the MAPK signaling in both control and *HS2ST1*-overexpressing cells, yet, the overall activation status of *HS2ST1*-overexpressing cells was lower than the controls (Figure 6B). To link the observed alterations in growth factor binding and mitogenic signaling to the phenotypic changes induced by altered *HS2ST1* expression, we evaluated cell viability and scratch wound repair in the absence or presence of the MAPK pathway (MEK1/MEK2) inhibitor U0126. siRNA depletion of *HS2ST1* resulted in increased viability of MDA-MB-231 (Figure 6C) and MCF-7 cells (Figure 6D), which was reduced to control levels by U0126, suggesting a role for MAPK signaling in the *HS2ST1*-dependent process. In addition, MAPK inhibition generated a phenocopy of the delay in scratch wound motility observed in *HS2ST1*-overexpressing MDA-MB-231 cells (Figure 6E). Overall,

these results indicated that altered cell behavior in *HS2ST1*-misexpressing breast cancer cells is associated with altered ligand binding and cellular signaling.

4 | DISCUSSION

In this report we demonstrate a novel role for *HS2ST1* in modulating breast cancer cell invasiveness via central signaling pathways including MAPK and Wnt (Figure 6F). Our findings are in line with studies in glioma, indicating a downregulation of *HS2ST1* and other HS sulfotransferases.⁵⁰ Mechanistically, HSPGs modulate MAPK signaling, promoting proliferation, migration, and invasion.^{45,51-53} In our model cell lines, this pathway is constitutively active in MDA-MB-231 cells, but not in MCF-7.^{51,54} Phosphorylation of Erk 1/2 (p44/42 MAPK) was less evident in *HS2ST1*-overexpressing MDA-MB-231 cells

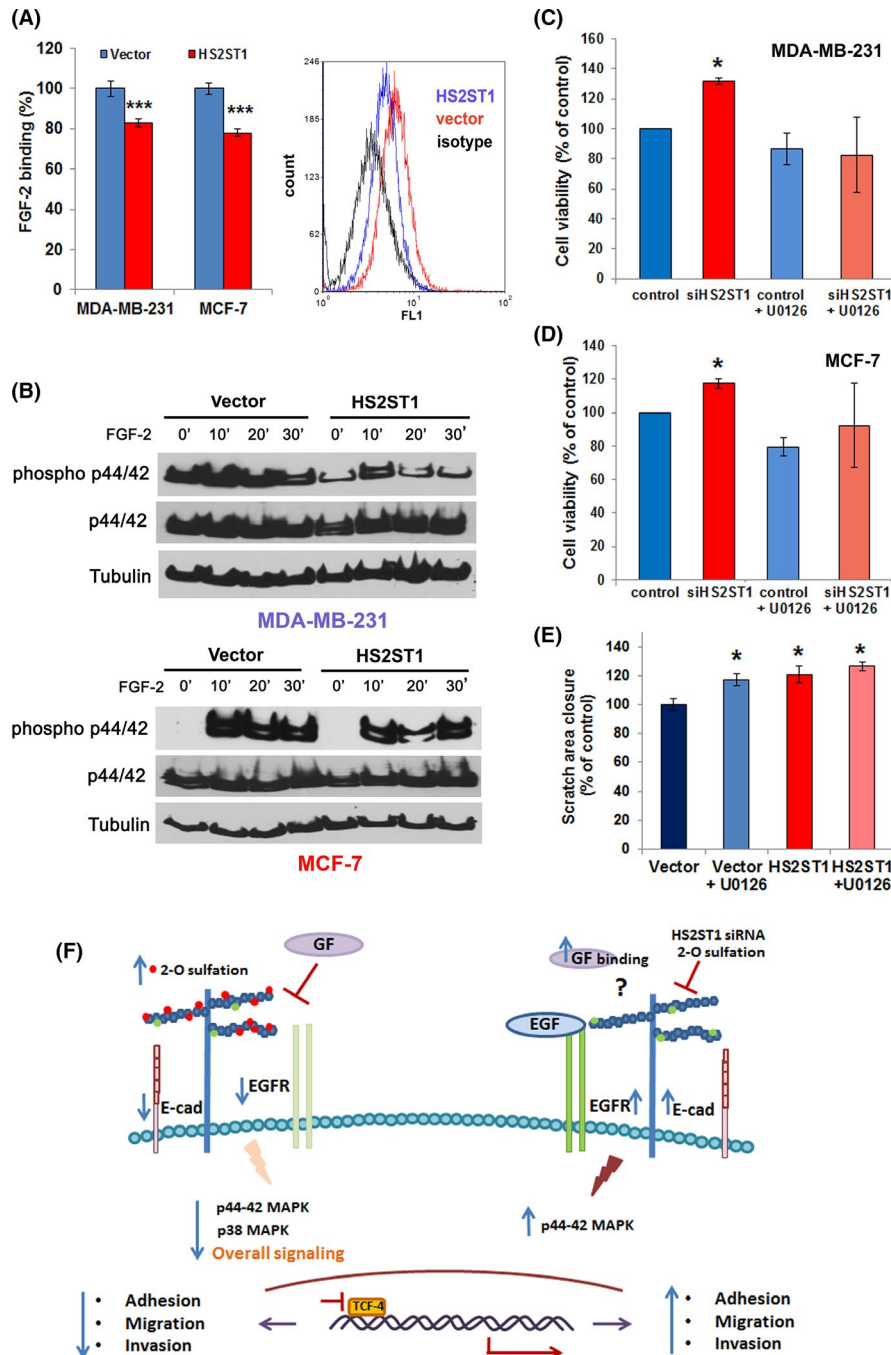


FIGURE 6 Altered *HS2ST1* expression affects FGF-2 binding, FGF-2-induced MAPK signaling, and MAPK-dependent cell viability and migration. A, *HS2ST1*-overexpressing breast cancer cells show reduced cell surface binding of biotinylated FGF-2. Flow cytometry, reveals a decrease of 20% and 30% in the binding efficiency of FGF-2 to *HS2ST1*-overexpressing MDA-MB-231 and MCF-7 cells compared with controls. Right panel, representative measurement (MCF-7 cells). *** $P < .001$, $N = 3$, error bars = SEM. B, *HS2ST1*-overexpression attenuates FGF-2-induced MAPK activation. Serum-starved vector and *HS2ST1*-transfected cells were stimulated with 20 ng/mL FGF-2 for a 0–30 min time course and 25 μ g of cell lysate/lane were analyzed by western blotting for phospho-p44/42, p44/42 and the loading control α -tubulin. C, D, *HS2ST1* siRNA knockdown enhances breast cancer cell viability in a MAPK-dependent manner. MDA-MB-231 (C) and MCF-7 (D) cells were subjected to control or *HS2ST1* siRNA treatment in the absence or presence of the MAPK inhibitor U0126. While *HS2ST1* siRNA knockdown significantly enhanced breast cancer cell viability, this effect could be reduced to control levels by MAPK inhibition. * $P < .05$, $N = 6$, error bars = SEM. E, *HS2ST1* overexpression and MAPK inhibition by the MAPK inhibitor U0126 delay scratch wound repair in MDA-MB-231 cells. MAPK inhibition generates a phenocopy of *HS2ST1* overexpression. * $P < .05$, $N \geq 3$, error bars = SEM. F, Model of the tumor-suppressive role of *HS2ST1* in breast cancer. High levels of *HS2ST1* resulted in structural changes in HS and altered growth factor binding, which resulted in attenuated signaling through the MAPK and additional pathways. Reduced signaling and expression of E-cadherin and EGFR was associated with reduced viability, adhesion, migration, and invasion of breast cancer cells

compared with the controls, correlating with the reduced invasive phenotype. In MDA-MB-231 cells, knockdown of *HS2ST1* did not result in a further increase in p44/42 MAPK activation, possibly because this pathway is already strongly constitutively active in these cells.⁵⁴ In contrast, in MCF-7 cells, *HS2ST1* knockdown was sufficient to activate this pathway, possibly because altered HS sulfation may have affected the binding and presentation of mitogens to a broad range of receptors, thus acting as a co-receptor and enhancing signaling through the MAPK pathway upon ligand engagement.^{1,2,4} Surprisingly, siRNA knockdown of *HS2ST1* did not enhance invasiveness of MCF-7 cells in our Matrigel chamber assay. It has been shown that stimulation of MCF-7 cells with growth factors such as TGF- α only leads to transient activation of MAPK signaling and cell migration, whereas the same induces sustained MAPK activation, enhanced cell motility, and increased in vitro invasion of MDA-MB-231 cells,⁵¹ which may be an underlying cause for our observation. Another MAPK, p38, was also less activated in *HS2ST1*-overexpressing MDA-MB-231 cells supporting the observed phenotype. Intriguingly, phosphokinase array data revealed an overall *HS2ST1*-dependent decrease in the phosphorylation status of p38, Erk 1/2, EGFR, Akt, JNK and several other proteins (see Table 2). The reduced overall signaling is in accordance with the observed decreased invasiveness of *HS2ST1*-overexpressing cells. EGFR, known to be overexpressed and activated in approximately 20%-25% of cancers,⁵⁵ acts as a central point for the activation and crosstalk with many signaling pathways relevant to tumor migration and invasion.⁵⁶ MAPK and other kinases like phosphatidylinositol-3-kinase (PI3-K) act downstream of EGFR activation, promoting tumor cell migration and invasion.^{50,57-59} In addition, decreased activation or inhibition of p38 MAPK decreases breast cancer metastasis.⁶⁰

Altered MAPK activation resulted in decreased expression of the Wnt pathway-related transcription factor Tcf4 (Tcf7/L2) in *HS2ST1*-overexpressing MDA-MB-231 cells. Indeed, a HS-dependent crosstalk between Wnt and MAPK pathways modulates invasiveness via regulation of several downstream targets such as proteases, cadherins, and ECM components.⁴⁵ Apart from Tcf4, Wnt 7a was transcriptionally upregulated in *HS2ST1*-overexpressing cells. Wnt signaling has been linked to the mammalian target of rapamycin (mTOR) pathway,⁶¹ which promotes cancer cell invasion.⁶² Notably, reduced mTOR signaling is associated with increased hormone resistance in MCF-7 cells,⁶³ while MAPK pathway activation may enhance the sensitivity of these cells to estradiol.⁶⁴ As the triple-negative cell line MDA-MB-231 does not express estrogen receptor alpha, differential activation of these pathways may have contributed to the partially divergent impact of *HS2ST1* on hormone receptor-positive and hormone receptor-negative cells. Previous studies have demonstrated the positive regulation of E-cadherin expression by Wnt 7a in lung cancer cells.⁶⁵ E-cadherin acts as a tumor suppressor because of its role in establishing cell-cell adhesion⁶⁶ and is known to be co-expressed along with syndecan-1 HSPG in early stages of breast cancer.⁴² Here, we show that *HS2ST1* overexpression directs up-regulation of E-cadherin, thereby establishing cell-cell adhesion,

while the knockdown suppresses its expression, which is associated with cancer progression and metastasis.⁶⁷ Upregulation of E-cadherin in *HS2ST1*-overexpressing MCF-7 cells may have also had an impact on Tcf-4, as the cytoplasmic domains of E-cadherin bind to beta-catenin,⁶⁸ which would then not be available to activate Tcf-4. Notably, decreased E-cadherin expression and loss of cell-cell adhesion were also observed in *HS2ST1* morphant cells of zebrafish embryos.⁶⁹ Reduction of cell-cell adhesion is accompanied by decreased cell adhesion to ECM ligands⁷⁰ and it is known that cell-cell and cell-matrix adhesion directly determine cell motility.^{2,35} We show a crucial role of *HS2ST1* in modulating cell-ECM adhesion as tumor cell-matrix adhesion is considered to be a key event regulating metastasis.^{36,71} Increased binding of *HS2ST1*-overexpressing cells to fibronectin and laminin correlates with the observed migration and invasion phenotypes suggesting that the altered surface structure (as determined by FTIR analysis) reduces ECM adhesion, thereby facilitating cell migration. This finding is supported by a previous study in which increased adhesion to laminin inhibited cell movement.⁷²

EGFR, one of the major upstream modulators of MAPK pathway is often overexpressed in more aggressive triple-negative breast cancers⁴⁶ and is associated with poor prognosis and large tumor size.^{73,74} EGFR acts as a central point for many signaling pathways, including G-protein coupled receptor signaling, which transactivates EGFR, inducing invasion.^{75,76} It regulates epithelial-to-mesenchymal transition via its downstream regulator-ERK inducing mesenchymal motility and invasion in cancer cells.⁷⁷ The *HS2ST1*-dependent changes in EGFR expression contribute to changes in invasive behavior of triple-negative MDA-MB-231 cells.

Previous studies have demonstrated that HS modulates signaling by growth factor binding^{25,49} and that 2-O-sulfated HS oligosaccharides effectively bind to FGF-2.⁴⁷ It has been shown that HS, which binds to FGF but does not bind to the fibroblast growth factor receptor (FGFR), acts as a competitor.¹⁷ We could demonstrate weaker binding of FGF-2 to *HS2ST1*-overexpressing cells, which may be one of the reasons for the observed reduction in signaling in these cells.^{78,79} Moreover, the formation of FGF-FGFR signaling complexes is generally believed to be promoted by HS chains that bind to both growth factors and receptors.^{80,81} The smaller effect of FGF-2 binding to cell surface HS compared with control cells could be due to steric hindrance of HS on the cell surface and/or to a conformational change in HS,⁸² compared with isolated HS, for which binding sites are more freely available. Mechanistically, this explains the overall phenotypic end points obtained as a result of modifying HS specifically at the 2-O position.

A novel and non-invasive approach for analyzing sulfated GAGs from CM led to good discrimination between control and *HS2ST1*-overexpressing cells. By focusing the analysis on the sulfate absorption region, *HS2ST1*-transfected cell CM was different from the control counterparts both for MDA-MB-231 and MCF-7 cells, although more marked for MDA-MB-231. This suggests that an FTIR spectroscopic approach could be used as a rapid monitoring method for screening CM.

In conclusion, this study provides evidence for the promising role of *HS2ST1* in modulating breast cancer cell invasiveness via modulating EGFR and additional signaling pathways (Figure 6F). This report is the first to reveal the impact of an HS modifier enzyme, *HS2ST1*, in controlling the invasiveness of breast cancer cells in vitro. Further in vivo and clinical studies in this direction will be of great interest and importance for future potential cancer therapeutics.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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