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FGF2 Binding, Signaling, and Angiogenesis Are Modulated by Heparanase in Metastatic Melanoma Cells¹

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Abstract

Heparanase (HPSE) and fibroblast growth factor-2 (FGF2) are critical regulators of melanoma angiogenesis and metastasis. Elevated HPSE expression contributes to melanoma progression; however, further augmentation of HPSE presence can inhibit tumorigenicity. HPSE enzymatically cleaves heparan sulfate glycosaminoglycan chains (HS) from proteoglycans. HS act as both low-affinity FGF2 receptors and co-receptors in the formation of high-affinity FGF2 receptors. We have investigated HPSE's ability to modulate FGF2 activity through HS remodeling. Extensive HPSE degradation of human metastatic melanoma cells (70W) inhibited FGF2 binding. Unexpectedly, treatment of 70W cells with low HPSE concentrations enhanced FGF2 binding. In addition, HPSE-unexposed cells did not phosphorylate extracellular signal-related kinase (ERK) or focal adhesion kinase (FAK) in response to FGF2. Conversely, in cells treated with HPSE, FGF2 stimulated ERK and FAK phosphorylation. Secondly, the presence of soluble HPSE-degraded HS enhanced FGF2 binding and ERK phosphorylation at low HS concentrations. Higher concentrations of soluble HS inhibited FGF2 binding, but FGF2 signaling through ERK remained enhanced. Soluble HS were unable to support FGF2-stimulated FAK phosphorylation irrespective of HPSE treatment. Finally, cell exposure to HPSE or to HPSE-degraded HS modulated FGF2-induced angiogenesis in melanoma. In conclusion, these effects suggest relevant mechanisms for the HPSE modulation of melanoma growth factor responsiveness and tumorigenicity.

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Keywords: Heparanase, heparan sulfate, FGF2 binding, angiogenesis, malignant melanoma.

biologic activity is mediated by their heparan sulfate glycosaminoglycan chains (HS) through the binding of a wide variety of growth factors, growth factor receptors, and adhesion molecules [2]. In addition, quantitative and qualitative changes occur in HS expressed by metastatic *versus* normal cells [2]. These include variations in chain sequence, sulfation, and length [2,3], leading to alterations in the ability of HS to modulate biologic activity [4].

HS sequence and length are regulators of fibroblast growth factor-2 (FGF2) activity, an important mediator of melanoma angiogenesis and progression [5]. HS bind to both FGF2 and FGF receptors (FGFR) [6]. Cell surface HSPG are low-affinity receptors for FGF2 through their HS. HSPG also form a ternary complex with FGF2 and their tyrosine kinase-containing FGFR, resulting in the formation of high-affinity binding sites [7,8]. Finally, soluble HS or the highly sulfated form of HS, heparin, can mediate the binding of FGF2 to FGFR, bypassing the need for cell surface HSPG [6].

FGF2 binding phosphorylates both extracellular signal-related kinase (ERK) and focal adhesion kinase (FAK), although not necessarily through the same signaling pathway [9,10]. Signal transduction pathways that include ERK are important regulators of cell growth and invasion and are altered during melanoma progression to metastatic phenotype [11,12]. ERK is also phosphorylated in response to a wide variety of extracellular signals, including adhesion molecules and growth factors, whereas FAK is activated during integrin binding and mediates signal transduction for biologic functions such as adhesion, migration, and survival [13]. For example, pathways that stimulate ERK phosphorylation in melanoma cells may then affect the production of angiogenic factors. ERK and FAK can also participate in shared signaling pathways (i.e., when integrin-induced FAK phosphorylation stimulates ERK signaling) [14]. However,

Abbreviations: DME/F-12, Dulbecco's modified Eagle's F-12 medium; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FGF2, fibroblast growth factor-2; FGFR, FGF receptors; Hep I, heparitinase I; Hep III, heparitinase III; HPSE, heparanase; HS, heparan sulfate glycosaminoglycan chains; HSPG, heparan sulfate proteoglycans

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Introduction

How cancer cells respond differentially to growth factors plays a major role in cancer progression. One family of molecules, heparan sulfate proteoglycans (HSPG)¹, is present both on the cell surface and in the surrounding extracellular matrix (ECM) and plays a major role in cellular behavior, leading to tumor growth and metastasis [1]. Much of HSPG

depending on the cellular system, ERK may be upstream of FAK [15], FAK may act as a potentiator of ERK signaling [16], or ERK may be independent of FAK activation [17,18].

HS remodeling by bacterial heparitinases modulates FGF2 binding, signaling, and subsequent activity. Depending on cell type and method of removal, degradation of cell surface HS can prevent the formation of high-affinity ternary complexes [19]. Free HS released by heparitinase can then act as either promoters or inhibitors of growth factor actions [20]. Conversely, FGF2 binding and activity can be affected to differing extents, particularly as ligand binding to a small number of receptors can stimulate a biologic response [21]. Heparanase (HPSE) has differential effects on growth factor activity, depending on the HS sequence it cleaves and the HS tissue origin. For example, soluble HS cleaved by bacterial heparitinase I (Hep I) promote FGF2 activity, whereas heparitinase III (Hep III)-cleaved HS do not [22].

Mammalian HPSE is an *endoglycosidase* (endo- β -D-glucuronidase), which degrades HS in a sequence-dependent manner [23]. It produces HS fragments larger than those produced by bacterial Hep I or Hep III enzymes; thus far, it has not been as well studied for its effects on growth factor activity. There are indications that HPSE activity is important in remodeling HS and in subsequent modification of growth factor activity (e.g., HPSE treatment of soluble syndecan-1, an important HSPG implicated in tumor biology, converts syndecan-1 from an inhibitor of FGF2 activity to a promoter of FGF2-stimulated mitogenesis) [24]. Early studies found that HPSE can increase the cellular availability of FGF2 by releasing sequestered FGF2 from the ECM into a bioactive form [2].

Of relevance, HPSE protein expression is upregulated in a wide variety of cancers [23], including malignant melanoma [25]. Overexpression of HPSE in transfected lymphoma [26] or melanoma cells [27] has been shown to augment tumor metastasis and angiogenesis. Likewise, inhibition of HPSE expression with antisense or siRNA techniques resulted in a significant decrease in melanoma metastatic phenotype [27,28]. However, it has been shown in a glioma model system that moderate levels of HPSE promoted tumor growth and invasion, whereas higher levels did not, indicating concentration dependence for its activity [29].

The present study focuses on HPSE's ability to both enhance and inhibit FGF2 binding and activity in an HPSE concentration-dependent manner. We studied HPSE-generated remodeling of both cell surface and soluble HS by determining how: 1) HPSE cleavage of cell surface HSPG modifies FGF2 binding and signaling; 2) HS produced by HPSE degradation modulate FGF2 binding and signal transduction; and 3) FGF2-induced angiogenesis is enhanced by HPSE and HPSE-generated HS at the same concentrations that augment FGF2 binding and signaling.

Materials and Methods

Tissue Culture and Enzymatic Treatments

Human 70W melanoma cells, which possess high metastatic abilities compared to MeWo parental counterparts [30],

were maintained as subconfluent monolayer cultures in Dulbecco's modified Eagle's F-12 medium (DME/F-12) supplemented with 10% (vol/vol) fetal bovine serum, as previously described [30]. Cells were washed thrice in DME/F-12 containing 0.1% bovine serum albumin (BSA) and penicillin/streptomycin, then incubated with indicated concentrations (0–5 μ g/ml) of recombinant HPSE in 10 mM HEPES-buffered DME/F-12 (pH 6.8) containing 0.1% BSA and penicillin/streptomycin, or with Hep III in 10 mM HEPES-buffered DME/F-12 (pH 7.4) containing 0.1% BSA and penicillin/streptomycin for 18 hours at 37°C. For Hep III-treated cells, an additional aliquot of enzyme was added on the last hour of incubation to counter possible enzyme inactivation. The above conditions were chosen for different enzyme optimum values for pH and time while maintaining experimental conditions as close as possible. Cells were washed thrice with DME/F-12 containing 0.1% BSA and penicillin/streptomycin before initiating the experiments.

HPSE Isolation and Activity

Recombinant human HPSE was purified as previously described [30,31]. Briefly, Sf9 insect cells, transfected with baculovirus transfer vectors containing HPSE subunits, were grown in SF900II serum-free medium (Gibco BRL, Grand Island, NY) for high-titer stocks. Tni cells cultured in suspension using ExCell405 serum-free medium (JRH Bioscience, Lenexa, KS) were infected with high-titer stock for 48 hours, and cells were subsequently removed by centrifugation. The supernatant was then tested for HPSE activity, filtered through a 0.45- μ m filter, and loaded on a HiTrap heparin column (Amersham Biosciences, Piscataway, NJ). The column was subsequently washed in Tris-buffered saline (TBS) then eluted using a 100-ml gradient of 0.15 to 1.0 M NaCl in 25 mM Tris-HCl (pH 7.5). Collected fractions (1 ml) were screened for HPSE activity (Heparan Degrading Enzyme Assay Kit; Takara Mirus, Madison, WI) [30]. HPSE eluted at 0.67 M NaCl, as expected [31].

Immunofluorescent Staining

70W cells were plated at 10% density in Lab-TekII chamberslides (Nalge Nunc International, Rochester, NY) in DME/F-12–10% (vol/vol) fetal bovine serum and allowed to attach and grow for 24 hours. Cells were treated with HPSE or Hep III as described above, and cell surface HS were detected using an anti-heparan sulfate monoclonal antibody (mAb) 10E4 (1:50 dilution; Seikagaku America, Falmouth, MA) followed by incubation with AlexaFluor 488 goat-anti-mouse IgM (1:400 dilution; Molecular Probes, Eugene, OR). Digital images were produced using an Axioplan fluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY) with Microfire digital camera (Optronics, Goleta, CA) and Picture-frame imaging program (Optronics) using identical conditions for all photographs.

Cellular HS Labeling and Degradation

70W cells were plated in 24-well tissue culture plates for 24 hours to allow cell attachment. Cells were labeled with 20 μ Ci/ml [³⁵S]sulfate (Amersham Biosciences) for 24 hours,

washed with DME/F-12 containing 0.1% BSA and penicillin/streptomycin, and treated with HPSE or Hep III, as described above. The medium was sampled at 0-hour time point to assure adequate washing. Conditioned medium was collected at 18 hours, added to Ultimate Gold (PerkinElmer Life and Analytical Sciences, Boston, MA) scintillation cocktail, and counted in a liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences). Cells were solubilized, and associated [³⁵S]sulfate was counted. Cell-associated and released counts were added for total [³⁵S]sulfate.

HS Degradation and Analysis

To degrade heparin or HS (bovine kidney HS; Sigma-Aldrich, St. Louis, MO), 250 ng of HS was incubated for 16 hours at 37°C with either HPSE (10 µg/ml) in 20 mM sodium acetate (pH 5.0), or Hep III (0.1 mg/ml) in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 4 mM CaCl₂ (final volume, 70 µl). Hep III-mediated degradation could be achieved in two 30-minute incubations; however, to mimic conditions for HPSE, a longer incubation time was maintained. Following digestion, enzymes were inactivated for 10 minutes at 100°C. Mock digestions were performed without enzymes as controls. Additional controls were performed using these enzymes without the presence of heparin or HS (data not shown).

HPSE-degraded HS used in binding and signaling experiments was analyzed by separating HS and heparin on a Criterion 4–20% TBE gel (Bio-Rad Laboratories, Hercules, CA) for 20 minutes at 60 mA. Bands were visualized with Alcian blue 8GX (Sigma-Aldrich), followed by silver staining (Pierce Endogen, Rockford, IL) [32]. Densitometric analyses were performed using a Versadoc imaging system (Bio-Rad Laboratories) to determine profiles' leading edge.

HPSE activity was determined by the degradation of a high-molecular-weight fraction of FITC-HS, which was prepared by employing the same HS used for binding and signal transduction experiments. FITC-HS was incubated for 0 to 24 hours with HPSE, as per above. After HPSE inactivation, the pH was adjusted using 0.5 M Tris (pH 8.0), and FITC-HS was analyzed using high-speed TSK gel permeation column chromatography [high-performance liquid chromatography (HPLC)], as previously described [30].

FGF2 Binding Assays

A widely accepted [¹²⁵I]FGF2 binding assay was used in our analyses [20,22]. Cells were plated in 24-well gelatin-coated tissue culture plates for 24 to 48 hours to allow cell adhesion. Cells were treated with HPSE or Hep III, as described above. Cells were washed thrice with binding buffer [HEPES-buffered DME/F-12 (pH 7.4) containing 0.2% BSA] at 4°C, and cells were subsequently assayed for [¹²⁵I]FGF2 (Amersham Biosciences) binding, as previously reported [20,33]. Briefly, cells were incubated for 3 hours with 200 pM [¹²⁵I]FGF2 in binding buffer at 4°C. They were then washed thrice with 20 mM HEPES (pH 7.4) containing 150 mM NaCl and 0.2% BSA at 4°C. Low-affinity HSPG binding sites were detected by two collected 1-ml washes of 20 mM HEPES (pH 7.4) containing 2 M NaCl and 0.2% BSA at 4°C. High-affinity FGFR complex binding sites were detected by two

collected 1-ml washes of 20 mM sodium acetate (pH 4.0) containing 2 M NaCl and 0.2% BSA at 4°C. Collected washes were counted in a Clinigamma counter (LKB Wallac, Gaithersburg, MD). For [¹²⁵I]FGF2 binding assays in the presence of soluble HS, [¹²⁵I]FGF2 and HS (0–1000 ng/ml) were added together, and assays were continued as described above. Control experiments were performed with unlabeled FGF2 to determine nonspecific binding. Results were reported as the relative binding of experimental condition compared to untreated or mock-treated controls. Significant differences in [¹²⁵I]FGF2 binding were determined by Student's *t* test.

Western Blot Analysis of ERK and FAK Phosphorylation

For signal transduction studies, cells were plated in 12-well tissue culture wells for 48 hours to allow cell adhesion. HPSE treatments of cell surface HS were performed as described above. Cells were exposed to 10 ng/ml FGF2 for 10 minutes at 37°C. Where indicated, FGF2 was added together with 0 to 1000 ng/ml HS. Following stimulation, the medium was aspirated from cells, and 100 µl of lysis buffer [20 mM Tris-HCl (pH 7.4) containing 1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.1 mM sodium molybdate, 1 mM sodium orthovanadate, and 5 µg/ml aprotinin] was added at 4°C. Lysed cells were scraped from the dish and put through three to six cycles of freezing in liquid nitrogen and heating up to 100°C for 5 minutes.

Samples for Western blot analysis were incubated at 100°C for 5 min with Laemmli sample buffer and separated on a 4–15% Criterion gel (Tris-HCl; Bio-Rad Laboratories). Proteins were transferred to polyvinylidene difluoride membrane (Pierce Endogen). Membranes were incubated in a blocking reagent [3% (wt/vol) nonfat dry milk and 0.5% (wt/vol) BSA in TBS] for 2 hours, and then a buffer was added for an additional 30 minutes. Membranes were then incubated for 16 hours in blocking reagent with one of the following primary antibodies: anti-phosphorylated FAK (tyrosine 397) polyclonal antibody (Biosource International, Camarillo, CA), anti-FAK polyclonal antibody, anti-ERK mAb, or anti-phosphorylated ERK mAb (Cell Signaling Technology, Beverly, MA). Membranes were washed for 1 hour with six to eight changes of TBS containing 0.5% IGE-PAL (CA-630; Sigma-Aldrich) and incubated with horseradish peroxidase-anti-mouse or anti-rabbit IgG (1:10,000 dilution; Accurate Chemical and Scientific Co., Westbury, NY). Membranes were then washed and developed using the Supersignal west femto maximum sensitivity substrate (Pierce Endogen). Labeling was detected and quantified using a Versadoc imaging system (Bio-Rad Laboratories).

Angiogenic Assays

Where indicated, cells were pretreated with HPSE or mock-treated, as per above. 70W cells were then released with a brief trypsin treatment and added to 1 mg/ml soybean trypsin inhibitor in DME/F-12 containing 0.1% BSA, washed twice in DME/F-12, and resuspended at 1×10^7 cells/ml in 50% reduced growth factor Matrigel (Becton Dickinson,

Labware, Bedford, MA) in DME/F-12 at 4°C. HPSE, HS fragments, and FGF2 (10 ng/ml) were added accordingly. With the use of a 25-gauge needle, cells (2×10^6) were injected to the left and right abdominal subcutaneous tissues of female nude mice (Harlan Teklan, Madison, WI). Mice were sacrificed on day 10, tumors were excised, fixed in 10% formalin, embedded in paraffin, and cut to 7- μ m sections, which were stained with hematoxylin and eosin (H&E). Blood vessel density was assessed by examining three independent areas in each of three independent sections. Significant differences in angiogenesis were determined by Student's *t* test and 90% confidence intervals, using Microsoft Excel (Microsoft Corporation, Redmond, WA).

Results

HPSE Action Remodels Cell Surface HS

The extent of HS remodeling on 70W by HPSE was assessed both by immunofluorescence and by the release of metabolically labeled HS into the culture medium. No detectable reduction in HS expression levels was seen by immunofluorescent labeling of HS after treating cells with 5 ng/ml HPSE *versus* no HPSE (Figure 1, A and B). When cells were treated with higher HPSE concentrations (50–500 ng/ml), a decrease in cell surface HS expression was observed (Figure 1, C and D). HS expression also diminished when cells were exposed to increasing Hep III concentrations (Figure 1, E and H). Comparable results were obtained when cells, whose HS were metabolically labeled with [35 S]sulfate, were treated with HPSE and then radio-labeled HS in the culture medium were monitored. Lower HPSE concentrations (50 ng/ml and below) did not result in significant HS in the medium (Figure 1I). However, HPSE effectively released HS at higher concentrations (100–2500 ng/ml). Hep III release of labeled HS was linear between 5 and 2500 ng/ml Hep III (Figure 1I). Although Hep III cleaves HS into smaller fragments, the degradation was not as complete, possibly due to the reincorporation of released HS into the cell.

HPSE Action Modulates FGF2 Binding

The integrity of HS on HSPG influences the binding of heparin-binding growth factors. To determine whether HPSE remodeling of HS affected FGF2 binding, 70W human melanoma cells were degraded with HPSE (0–2500 ng/ml) and assayed for FGF2 binding to low-affinity (HS) and high-affinity (FGFR ternary complex) sites [20,22]. Inhibition of low-affinity sites was detected at 50 ng/ml HPSE (24% inhibition; $P < .005$) and maximal inhibition (59% inhibition; $P < .005$) was observed using 2.5 μ g/ml HPSE (Figure 2A). At the highest HPSE concentration (2.5 μ g/ml), high-affinity binding was inhibited by 48% ($P < .005$); thus, HPSE cleaved sufficient cell surface HS to inhibit high-affinity FGF2 binding (Figure 2A). Notably, HPSE concentrations that did not inhibit binding to low-affinity sites (5 ng/ml) enhanced FGF2 high-affinity binding by 157% ($P < .01$) compared to non-HPSE-treated cells (Figure 2A).

Either the extent of HS degradation by enzymatic degradation or differences in sequence cleavage could affect FGF2 binding. Therefore, 70W cells were treated with Hep III, a bacterial exoglycosidase that extensively cleaves HS at sites

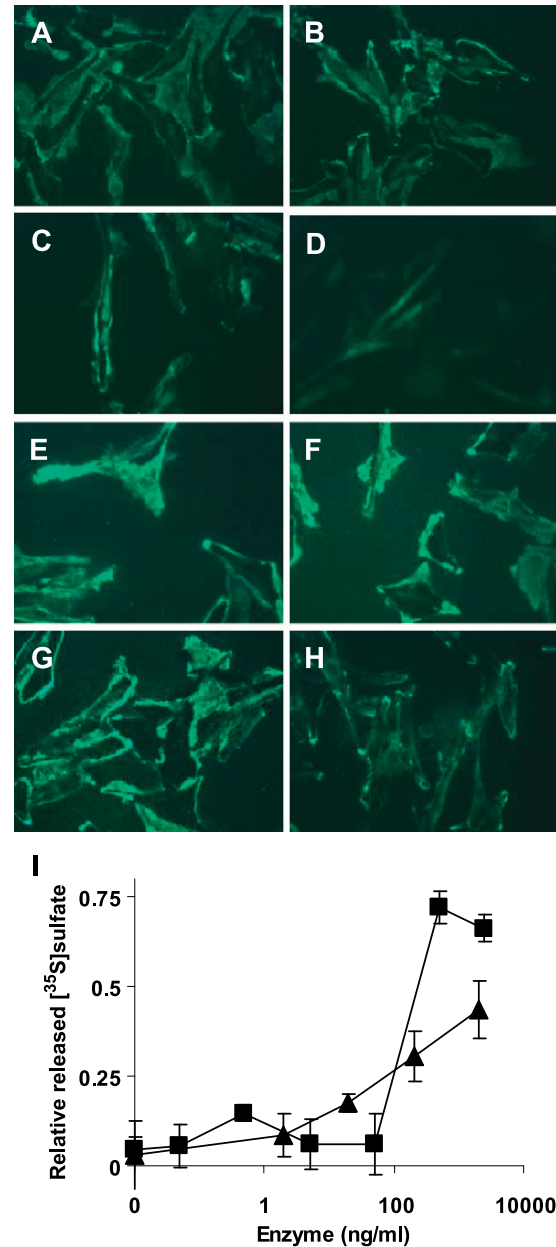


Figure 1. HPSE and Hep III degradation of cell surface HS (A–H). Human 70W melanoma cells, plated in chamber slides, were treated with (A) 0 ng/ml HPSE, (B) 5 ng/ml HPSE, (C) 50 ng/ml HPSE, (D) 500 ng/ml HPSE, or (E) 0 ng/ml Hep III, (F) 5 ng/ml Hep III, (G) 50 ng/ml Hep III, and (H) 500 ng/ml Hep III. Cells were then fixed and immunostained for HS. All images were produced at identical conditions using an Axioplan fluorescent microscope with Microfire digital camera and Pictureframe imaging program (see Materials and Methods section for details). (I). Alternatively, 70W cells were metabolically labeled for 24 hours with 20 μ Ci/ml [35 S]sulfate, washed to remove free sulfate, and incubated for 16 hours with indicated concentrations of HPSE (■) or Hep III (▲). Conditioned medium and cell-associated [35 S]sulfate were counted in a liquid scintillation counter and reported as mean fraction of [35 S]sulfate released into the medium compared to mean total [35 S]sulfate (see Materials and Methods section for details) from triplicate determinations (\pm standard deviations).

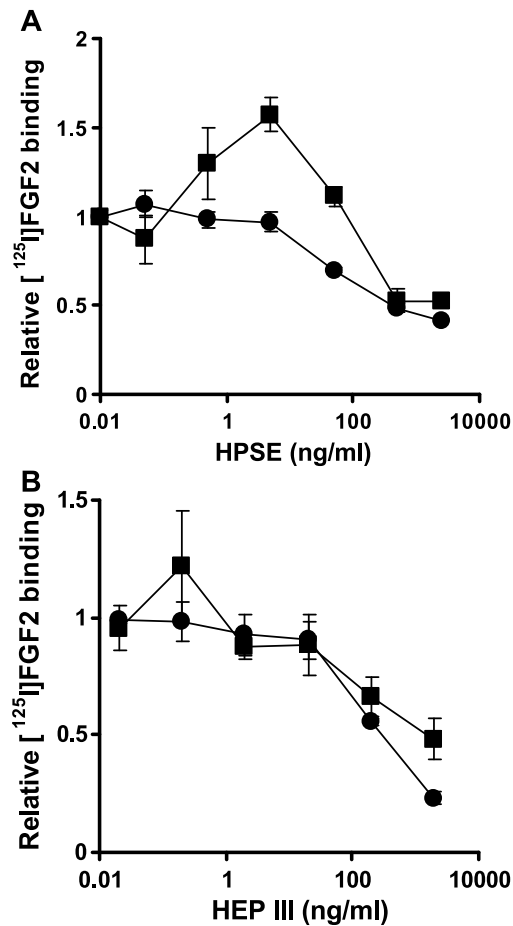


Figure 2. FGF2 binding of HPSE-treated or Hep III-treated human 70W melanoma cells. Cells were incubated for 16 hours with indicated concentrations of HPSE (A) or Hep III (B), washed to remove free HS, and incubated for 3 hours with [¹²⁵I]FGF2. Bound [¹²⁵I]FGF2 was separately released from low-affinity (●) and high-affinity (■) sites, and radioactivity was counted using an LKB gamma counter. Binding is reported as the mean [¹²⁵I]FGF2 bound to experimental conditions relative to the mean [¹²⁵I]FGF2 bound to untreated cells from triplicate determinations (\pm standard deviations).

differing from HPSE and known to inhibit FGF2 binding on other cell types [19]. Low-affinity FGF2 binding to Hep III-exposed 70W cells was maximally inhibited (77% inhibition; $P < .005$) at 2.5 μ g/ml enzymatic concentrations (Figure 2B). Likewise, Hep III degradation inhibited high-affinity FGF2 binding with maximal inhibition at 2.5 μ g/ml Hep III (52% inhibition; $P < .005$). However, unlike HPSE, Hep III treatment did not significantly enhance FGF2 binding to high-affinity sites (Figure 2B).

HPSE Regulates FGF2 Signal Transduction by Degrading Cell Surface HS

To determine whether FGF2 signal transduction depends on HPSE treatment of cell surface HSPG, we assessed FGF2-stimulated phosphorylation of molecular components known to be activated during FGF2 signaling. FAK and ERK are phosphorylated by different mechanisms on FGF2 stimulation [18,21,34]. 70W cells were exposed to FGF2 following cellular treatment with increasing HPSE concentrations. Untreated cells did not phosphorylate ERK in response to

FGF2 (Figure 3). However, ERK phosphorylation was increased in FGF2-stimulated cells following 0.5 ng/ml HPSE treatment. Exposure of cells to 5 ng/ml HPSE further enhanced FGF2-mediated ERK phosphorylation (Figure 3). Higher HPSE concentrations (5000 ng/ml) eliminated FGF2 stimulation of ERK phosphorylation (Figure 3). The higher HPSE concentration (5000 ng/ml) was used because 500 ng/ml HPSE reduced FGF2-stimulated ERK phosphorylation but did not abolish FGF2 response (data not shown). Furthermore, FGF2-stimulated FAK phosphorylation occurred only in 70W cells after HPSE treatment (Figure 3). Although FGF2 did not affect FAK phosphorylation in untreated cells, FGF2 stimulated FAK phosphorylation in cells treated with 0.5 to 5 ng/ml HPSE (Figure 3). As with ERK, HS degradation with 5000 ng/ml HPSE did not support FGF2-stimulated FAK phosphorylation. Furthermore, FGF2-mediated ERK and FAK phosphorylation (Figure 3) occurred at HPSE concentrations that increased FGF2 binding (Figure 2B).

HPSE-Degraded HS Modulates FGF2 Binding

Sulfation patterns of HS are cell type-specific, and these patterns have been shown to influence both HPSE activity and FGF2 binding [20]. To define HPSE enzymatic cleavage of HS and heparin used for FGF2 binding and signaling, we first analyzed HS and heparin fragments produced following HPSE treatment. Heparin or HS were incubated with HPSE for 16 hours, and initial assessment of degradation was determined by gel electrophoresis. Because HS were a heterogeneous mixture, they migrate as a broad band during gel electrophoresis (Figure 4A). HPSE-mediated HS degradation resulted in a migration shift, indicating a reduction in HS fragment size (Figure 4A). This shift was small as expected because HPSE does not extensively cleave HS. As control, HS were treated with Hep III, which results in much smaller fragments as indicated by their increased mobility. As expected, HPSE and Hep III degradation of heparin resulted in a shift in migration smaller than the one observed for HS (Figure 4, A and B). The leading edge of HS and heparin profiles was determined after gel densitometric analysis

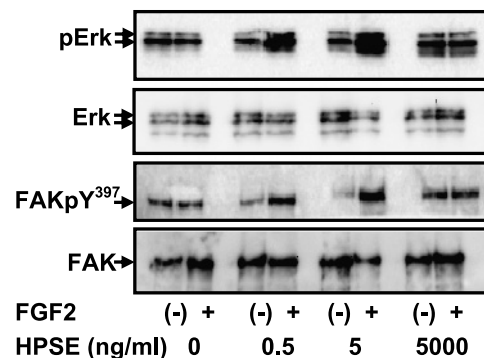


Figure 3. FGF2 signaling in HPSE-treated human 70W melanoma cells. Cells treated with 0 to 5000 ng/ml HPSE for 16 hours were washed, stimulated for 10 minutes with FGF2 (10 ng/ml), and lysed. Total cell lysates were then analyzed by Western blot analysis for total and phosphorylated ERK and FAK proteins (see Materials and Methods section for details). Results are representative of three independent experiments.

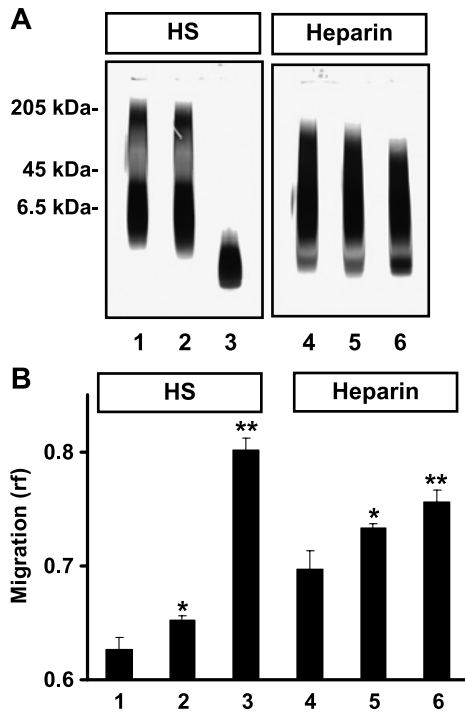


Figure 4. HPSE degrades HS and heparin. To assess HPSE-produced fragments, HS (lanes 1–3) or heparin (lanes 4–6) was incubated for 16 hours at 37°C with either buffer alone (lanes 1 and 4), 10 μ g/ml HPSE (lanes 2 and 5), or 100 μ g/ml Hep III (lanes 3 and 6). HS and heparin were then separated on a 4% to 20% TBE gel, and bands were visualized using Alcian blue dye followed by silver staining (A). Densitometric analysis of the above gel electrophoresis used a Versadoc imaging system (B). Migration is reported as the relative front (rf) of the leading edge of profiles. Means and standard deviations were determined from triplicate HS profiles. Significant differences were assessed by Student's *t* test (**P* < .03, ***P* < .005).

(Figure 4B) and demonstrated consistent shifts toward lower-molecular-weight HS with HPSE treatment compared to non-HPSE-degraded controls (*P* < .03). To further estimate reaction completion, a time course of HPSE degradation of high-molecular-weight HS was analyzed by HPLC. HS was fluorescently labeled with FITC, and then column chromatography was used to isolate the high-molecular-weight fraction. This was subsequently degraded with HPSE for varying times, and the extent of degradation was assessed using HPLC. A 4-hour HPSE treatment degraded 64% of initial HS (Table 1), as demonstrated by the profile shift to the right when compared to the non-HPSE-treated HS profile. However, retention time did not show a large shift, indicating that degradation at 4 hours was not complete (Table 1). Conversely, at 16-hour HPSE incubation, both profiles (84% degraded HS) and retention time shifts of FITC HS were maximal, indicating reaction completion (Table 1).

We then assessed FGF2 binding in the presence of heparin or bovine kidney HS degraded with or without HPSE. Figure 5 shows that both HPSE-degraded HS fragments from heparin (Figure 5, A and B) and bovine kidney HS (Figure 5, C and D) inhibited FGF2 low-affinity binding to 70W melanoma cells at high (> 10 ng/ml) concentrations. Inhibition of low-affinity binding was similar with HPSE-degraded or intact (nondegraded) heparin (Figure 5A) and HS (Figure 5C),

although HPSE-degraded heparin was slightly more effective at inhibiting low-affinity binding at low concentrations than intact heparin or HS preparations. Similarly, both HS and heparin inhibited high-affinity FGF2 binding at high concentrations. HPSE degradation of HS or heparin was not required for this binding inhibition. This binding was enhanced by the addition of low concentrations of HPSE-degraded HS or heparin compared to control heparin (Figure 5B) or HS (Figure 5D). Therefore, HPSE altered HS and heparin; thus, there was an increased high-affinity FGF2 binding at HS concentrations that did not affect low-affinity binding.

HPSE-Degraded HS Modulates FGF2 Signal Transduction

Because HS length and sulfation patterns contributing to FGF2 signal transduction can differ from those required for binding, we examined the ability of HPSE-degraded HS to facilitate FGF2-stimulated ERK and FAK phosphorylation. As observed above, FGF2 failed to stimulate ERK phosphorylation in untreated 70W cells (Figure 6, A and B); however, incubation of cells with HPSE-degraded HS recovered FGF2-stimulated ERK phosphorylation (Figure 6A). Likewise, control HS enhanced FGF2-stimulated ERK phosphorylation at all considered concentrations (1–1000 ng/ml; Figure 6B). FGF2-stimulated ERK phosphorylation was robust by several criteria. First, treatment of cells with 1000 ng/ml HPSE-degraded HS (Figure 6A) effectively promoted FGF2-stimulated ERK phosphorylation despite the inhibition of FGF2 binding (Figure 5, C and D). This is not completely unexpected as FGF2 activity has been previously found to be more difficult to inhibit than FGF2 binding [35,36]. Second, control HS promoted FGF2-stimulated ERK phosphorylation (Figure 6B), although it did not augment FGF2 binding (Figure 5C). Finally, FAK was not phosphorylated in response to FGF2 in control 70W cells (Figure 6, A and B) as was also observed in the HPSE degradation curve (Figure 3). The addition of intact or HPSE-degraded HS did not recover FGF2-stimulated FAK phosphorylation (Figure 6, A and B), unlike the one detected in response to FGF2 in HPSE-exposed cells (Figure 3).

HPSE and HPSE-Degraded HS Modulate FGF2-Induced Angiogenesis

To determine whether degradation of cell surface HS modifies tumor-induced angiogenesis, 70W cells were exposed to increasing HPSE concentrations and injected subcutaneously into athymic nude mice with FGF2 and HPSE. Tumors were excised after 10 days and examined for blood

Table 1. HPLC Analysis of HS Digested by HPSE.

HS Digestion Time (hr)	Retention Time (min)	% Digested HS*
0	13.5	0
4	13.9	64
8	16.2	78
16	19.1	84
24	19.2	88

*The percentage of digested HS is the amount of HS HPLC profiles shifted out of nontreated control profiles divided by the total HS present.

vessel formation. FGF2 enhanced melanoma blood vessel formation by 2.3- to 3.6-fold (Figure 7). Treatment of melanoma cells with 10 ng/ml HPSE enhanced FGF2-induced angiogenesis compared to FGF2 with no HPSE ($P < .05$; Figure 7). Higher HPSE concentrations (1000 ng/ml) did not increase FGF2-induced angiogenesis above controls (FGF2, no HPSE). Treatment of FGF2-stimulated 70W cells with 10 ng/ml HPSE resulted in augmented angiogenesis compared to either no HPSE ($P < .05$) or cells treated with 1000 ng/ml HPSE ($P < .005$).

To define whether HPSE-degraded HS enhanced FGF2-induced angiogenesis, melanoma cells treated with HPSE-degraded HS, or mock-degraded intact HS, were subcutaneously injected into athymic nude mice in the presence/absence of FGF2. FGF2 did not stimulate additional angiogenesis above basal levels in melanoma tumors injected without HS (Figure 8). This may be due to higher basal levels of angiogenesis in these experiments compared to ones without enzyme pretreatment (Figure 7). This is possibly due to the conditions used for enzyme pretreatment. Melanoma tumors treated with HPSE-degraded HS and FGF2 had a significant increase in blood vessel density compared to either no FGF2 ($P < .005$) or FGF2 but no HS treatments ($P < .03$). Furthermore, the addition of intact HS with FGF2 did not result in augmented angiogenesis above basal levels.

Discussion

We report first-time evidence that HPSE alters metastatic melanoma cell responsiveness to FGF2, including FGF2 binding, signaling, and FGF2-induced angiogenesis. This provides a mechanism by which metastatic melanoma cells can become more responsive to FGF2, a growth factor that is important in melanoma progression. We have shown that HPSE degradation of cell surface HS can both augment and inhibit FGF2 activity, depending on the HPSE concentrations used to remodel cell surface HS. We have demonstrated a specificity of HPSE-modulated FGF2 increase in binding and signaling because, when we exposed cells to Hep III, a bacterial endoglycosidase, we observed a dose-dependent inhibition of FGF binding and signaling; however, there was no detectable increase. We also found that HPSE degradation of soluble heparin or HS produces fragments that either potentiate or inhibit melanoma cell FGF2 binding and signaling in an HS concentration-dependent manner. In addition, our results demonstrate that FGF2-mediated signaling pathways are differentially affected depending on whether cleaved HS are: 1) attached to cell surface HSPG or 2) in soluble form. Although we found that HPSE is able to stimulate FGF2-mediated angiogenesis, high levels of HPSE, which cause extensive HS degradation, are unable to support FGF2-mediated angiogenesis. Finally, we found that HPSE-produced HS fragments are able to promote FGF2-

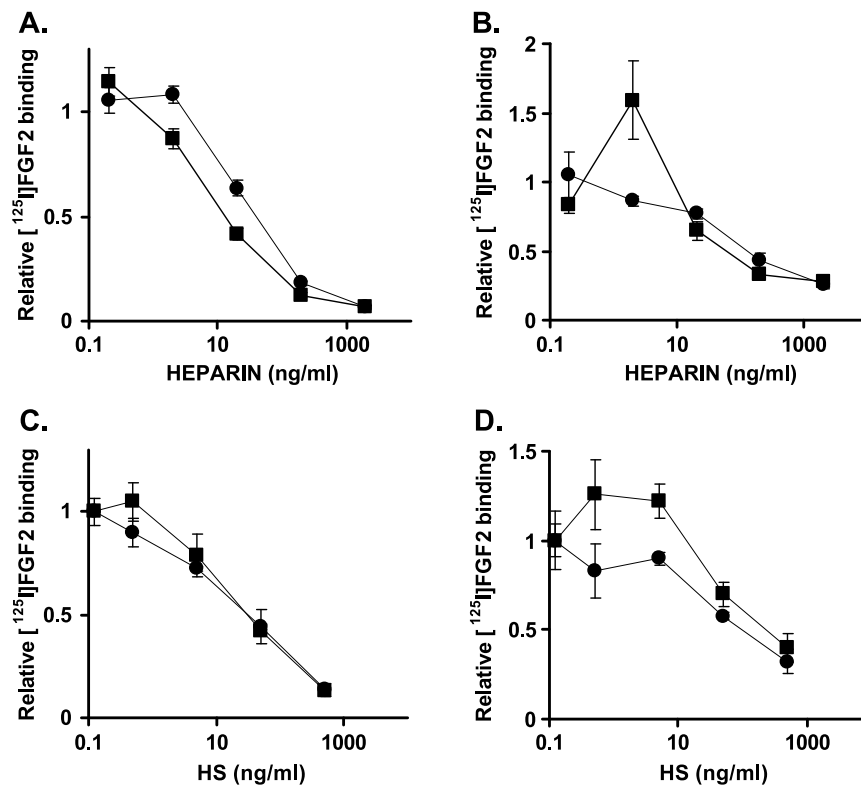


Figure 5. HPSE degradation of heparin or HS produces fragments that modulate FGF2 binding. Human 70W melanoma cells were incubated with [¹²⁵I]FGF2 and with indicated concentrations of heparin (A and B) or HS (C and D), which was predegraded with HPSE (■) or was mock-degraded (●). Samples were then assayed for [¹²⁵I]FGF2 binding to low-affinity (A and C) or high-affinity (B and D) sites. Binding is reported as the mean [¹²⁵I]FGF2 bound with experimental conditions relative to the mean [¹²⁵I]FGF2 bound to untreated control cells from triplicate determinations (\pm standard deviations).

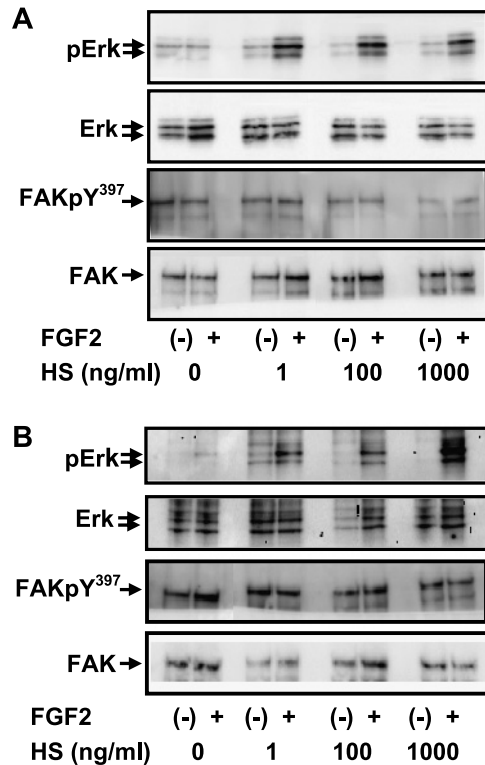


Figure 6. HPSE degradation of heparin or HS produces fragments that modulate FGF2-stimulated Erk but not FAK phosphorylation. Cells were incubated for 10 minutes with FGF2 (10 ng/ml) in the presence of indicated concentrations of HS predegraded with HPSE (A) or mock-degraded (B). Cells were lysed and total cell extracts were analyzed by Western blot analysis for total/phosphorylated forms of ERK or FAK, respectively (see Materials and Methods section for details). Results are representative of three independent experiments.

stimulated tumor angiogenesis in the complete absence of HPSE. Altogether, these results provide a relevant mechanism to elucidate how HPSE regulates tumor angiogenesis.

The high-affinity ternary complex includes FGF2 and HS as well as FGFR, which initiates signal transduction through its tyrosine kinase domain [37]. Structural analysis indicates that HS interacts with both FGF and FGFR in forming the high-affinity ternary complex [38,39]. However, HS requirements for binding and signaling are not equivalent [40]. Native HS on untreated 70W cells does not support FGF2-stimulated signaling, although it sustains binding. However, HPSE can modify cellular HS to support FGF2-stimulated signaling, potentially through modifying HS structures to alter interactions with either FGF2 or FGFR, or both. HS interactions can promote FGF2–FGFR dimerization initiating transphosphorylation of the receptor and signaling, as correct stoichiometry is required for FGF2 signaling [37,41]. Alternatively, HS may affect additional signal pathways required for efficient propagation of FGF2 signaling [6,42].

High-affinity FGF2 binding and signaling require HS sequence-specific interactions. Depending on the extent of HS degradation, HS sequences, which bind to either FGF2 or FGFR, could be removed or cryptic sites could be revealed [24,43]. HS are highly heterogeneous oligomers due to exten-

sive postsynthesis modification of the basic GlcUA_β_{1,4}GlcNAc unit. HPSE cleaves HS within a specified minimum sequence (–GlcNAc(6S)–GlcUA–GlcN(NS)–)₃ but also requires additional sulfation in the surrounding sequence [44]. Sites for HPSE cleavage occur infrequently in HS from multiple sources, producing long fragments capable of promoting FGF2 activity. There may also be additional sites of cleavage [45] that are suppressed in highly sulfated regions [44]. The HPSE cleavage sequence in HS does not overlap with the FGF2 HS binding site, which requires 2-*O* sulfation in the sequence HexA(2SO₄)–GlcNSO₃ for binding [46,47]. Highly sulfated regions are more effective at FGF2 binding as multiple 2-*O* sulfation increases binding [48,49]. In addition, HS binding to the FGFR requires 6-*O* sulfation, which is critical for strengthening FGF–FGFR binding and promoting receptor dimerization [40]. Conversely, Hep III produces HS fragments that are rich in IdoUA(2S)_α_{1,4}GlcNSO₃(±6S) disaccharides [50], and HS degradation is significantly more extensive than with HPSE, resulting in the production of shorter HS fragments. Although highly sulfated HS with as few as four disaccharides units can bind to FGF2, longer oligomers are required for effective formation of the FGF2–FGFR stoichiometry required for subsequent signaling [51].

HPSE cleavage of HS can potentially change core protein membrane localization, consequently altering their availability to interact with FGFR or other signaling molecules. This has been demonstrated with the HSPG syndecan-1 [52] and glypican [53], whose localization in the plasma membrane is affected by removing HS or by inhibiting HS interactions.

HPSE can release soluble HS from both cell surface and ECM HSPG and therefore alter the composition of soluble HS available to the cell. Previously, it has been shown that HPSE degradation of ECM releases HS with FGF2 in a bioactive form [23]. It has also been demonstrated that HS produced by bacterial heparitinases promoted tumorigenesis, depending on HS sequence specificity of the enzyme. We now demonstrate that HPSE, a unique mammalian HS degradative enzyme that is present during tumor progression and in normal physiological events, produces HS, which potentiate or inhibit FGF2 binding and signaling according to HS concentration and the signaling intermediate. In addition, these HS also promote tumor angiogenesis. Potentiation of FGF2 binding is more pronounced with HPSE-degraded heparin, possibly because the increased sulfation on heparin presents more FGF2 or FGFR binding sites. Enhanced binding and ERK signaling could result from soluble HS, which are more efficient than endogenous HS in the formation of the ternary FGFR complex. It is unexpected that shorter HS formed by HPSE degradation enhance ternary complex formation, whereas longer intact HS do not. Again, this suggests that HPSE cleavage may reveal cryptic binding sites or changes in HS concentration and length that influence the stoichiometry of the ternary complex.

In the melanoma cells used in this study, FGF2-stimulated FAK phosphorylation required HPSE remodeling of resident HSPG, and soluble HS did not recover FAK phosphorylation (Figures 2 and 4). FGF2 signaling involves cross-talk or stimulation with other signal transduction pathways [54].

For example, FGF2 can increase FAK phosphorylation initiated through integrins in an HS-dependent manner [21]. HPSE can activate integrins, which are known to phosphorylate FAK [29]. Therefore, in our cells, FAK may be sufficiently downstream of FGF2 that it requires activation of additional pathways for phosphorylation [9]. HSPG roles are of further interest because reducing the number of HS attached to a single core protein can inhibit the ability of HSPG to promote cell adhesion and invasion [55]. This would subsequently inhibit signaling dependent on these adhesive interactions.

We also found a modulation of tumor angiogenesis by HPSE and HPSE-degraded HS. Angiogenesis is dependent on multiple components that can be affected by HPSE. HS in the ECM provide binding sites for angiogenic factors such as FGF2 and vascular endothelial growth factor. Cell surface HSPG acts as growth factor and adhesion receptors on tumor cells and vascular endothelial cells. Modifying the HS may affect tumorigenicity by modifying the responsiveness of multiple receptors to the extracellular environment. In addition, HPSE releases HS from the ECM, which can alter binding and signaling initiated through endothelial or tumor HS binding cell surface receptors.

Finally, recent evidence suggests that, although HPSE upregulation in glioma cells results in augmented tumor

aggressiveness, further elevation of HPSE inhibits tumorigenicity [29]. Our work not only extends this notion to metastatic melanoma, suggesting that HPSE modulates tumor responsiveness in multiple systems, but also demonstrates that these changes can be affected by HPSE-mediated HS remodeling of the tumor cell surface and the ECM. Extensive degradation of cell surface HS can interfere with growth factor binding and internalization [2,3]. However, a modest degradation of cell surface HS could modify the structure of HS, resulting in either decreased or increased interactions depending on the growth factor. Furthermore, HPSE degradation of HS could change the availability of FGF2 in the tumor microenvironment by releasing FGF2 from the matrix and the cell surface. In addition to modifying FGF2 action, HPSE could alter signaling initiated by multiple heparin binding growth factors, fine-tuning cellular response to changes in its environment. Besides FGF2, melanoma cells respond to heparin binding growth factors, such as pleiotrophin and vascular endothelial growth factor, whose binding and signaling are also influenced by available HS [56,57]. This in turn can allow differing modifications of various biologic responses, such as angiogenesis or metastasis, depending on growth factors' availability and the amount of HPSE present. It will be of interest to determine HPSE effects on the

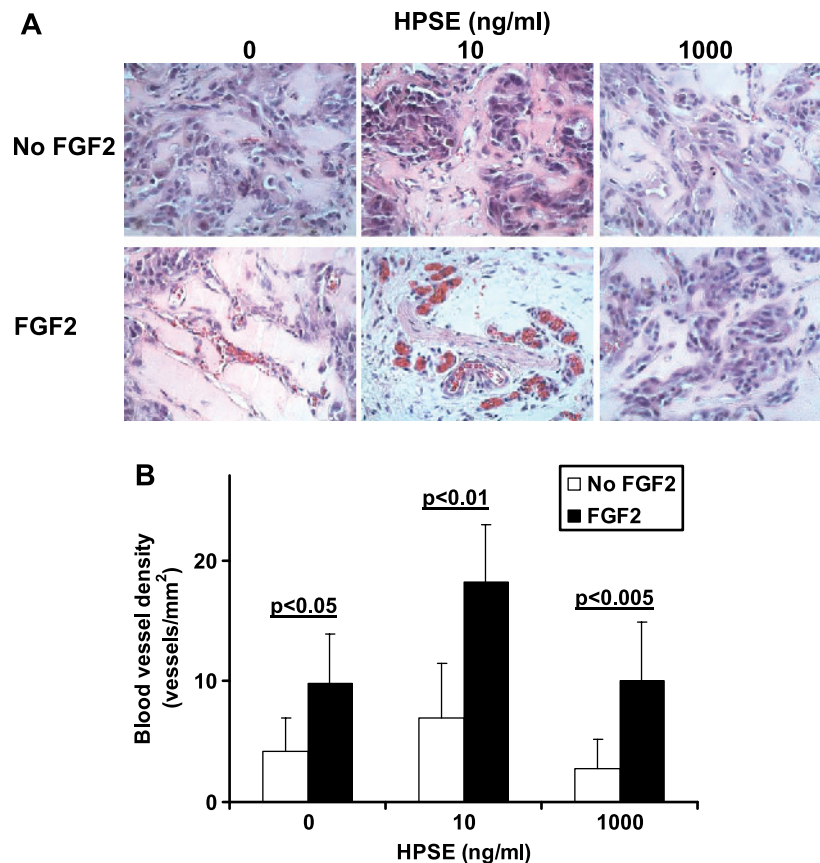


Figure 7. HPSE degradation of melanoma cell surface HS modulates FGF2-induced tumor angiogenesis. 70W cells were incubated for 16 hours with indicated HPSE concentrations. Cells were then released, resuspended with Matrigel in the presence or absence of FGF2, and injected subcutaneously into athymic nude mice ($n = 10$). Tumors were allowed to grow for 10 days, excised, and formalin-fixed, and sections were stained with H&E (A). Blood vessels were quantified using nine fields each of three independent sections per tumor (B). Original magnification, $\times 40$. Bar, 25 μm . Errors are reported at 90% confidence intervals.

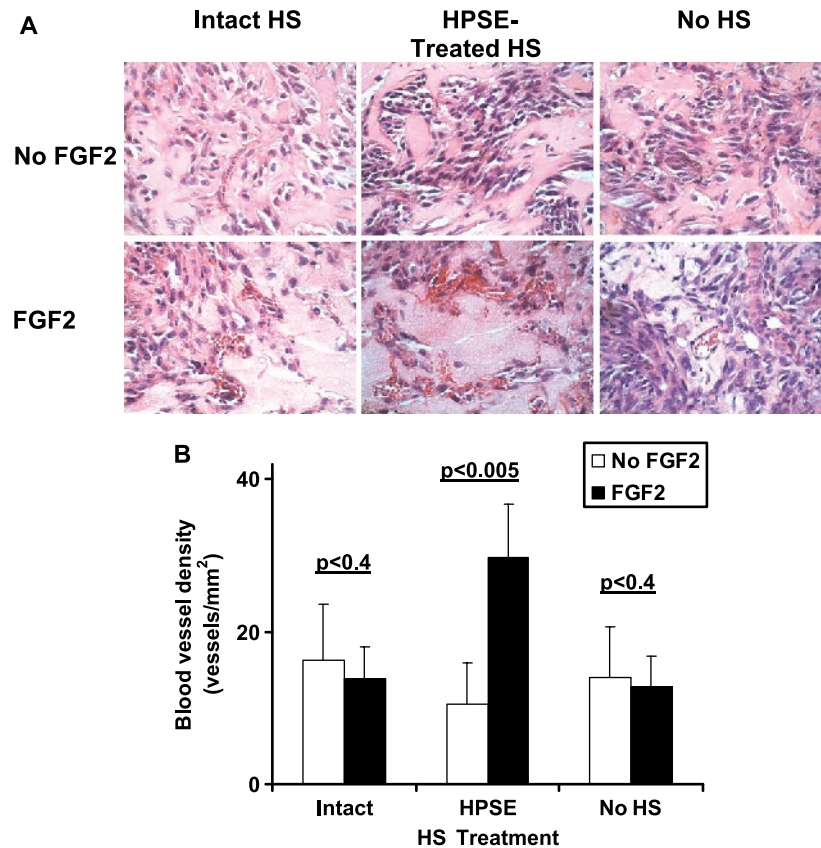


Figure 8. HPSE-degraded HS modulate FGF2 stimulation of melanoma tumor angiogenesis. Cells were released, resuspended with Matrigel in the presence or absence of FGF2 and indicated HS, and injected into athymic nude mice subcutaneously ($n = 8$). Tumors were allowed to grow for 10 days, excised, and formalin-fixed, and sections were stained with H&E (A). Blood vessels were quantified using nine fields each of three independent sections per tumor (B). Original magnification, $\times 40$. Bar, 25 μm . Errors are reported at 90% confidence intervals.

binding and signaling of these and other heparin binding growth factors and their related receptors, thus amplifying our knowledge of HPSE-based mechanisms of action.

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