Quantitative Phosphoproteomics Analysis Reveals Broad Regulatory Role of Heparan Sulfate on Endothelial Signaling*§

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Heparan sulfate (HS) is a linear, abundant, highly sulfated polysaccharide that expresses in the vasculature. Recent genetic studies documented that HS critically modulates various endothelial cell functions. However, elucidation of the underlying molecular mechanism has been challenging because of the presence of a large number of HS-binding ligands found in the examined experimental conditions. In this report, we used quantitative phosphoproteomics to examine the global HS-dependent signaling by comparing wild type and HS-deficient endothelial cells that were cultured in a serum-containing medium. A total of 7222 phosphopeptides, corresponding to 1179 proteins, were identified. Functional correlation analysis identified 25 HS-dependent functional networks, and the top five are related to cell morphology, cellular assembly and organization, cellular function and maintenance, cell-to-cell communication, inflammatory response and disorder, cell growth and proliferation, cell movement, and cellular survival and death. This is consistent with cell function studies showing that HS deficiency altered endothelial cell growth and mobility. Mining for the underlying molecular mechanisms further revealed that HS modulates signaling pathways critically related to cell adhesion, migration, and coagulation, including ILK, integrin, actin cytoskeleton organization, tight junction and thrombin signaling. Intriguingly, this analysis unexpectedly determined that the top HS-dependent signaling is the IGF-1 signaling pathway, which has not been known to be modulated by HS. In-depth analysis of growth factor signaling identified 22 HS-dependent growth factor/cytokine/growth hormone signaling pathways, including those both previously known, such as HGF and VEGF, and those unknown, such as IGF-1, erythropoietin, angiopoietin/Tie, IL-17A and growth hormones. Twelve of the identified 22 growth factor/cytokine/growth hormone signaling pathways, including IGF-1 and angiopoietin/Tie signaling, were alternatively confirmed in phospho-receptor tyrosine kinase array analysis. In summary, our SILAC-based quantitative phosphoproteomic analysis confirmed previous findings and also uncovered novel HS-dependent functional networks and signaling, revealing a much broader regulatory role of HS on endothelial signaling. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.026609, 2160–2173, 2013.

Heparan sulfate (HS) is a linear, highly sulfated polysaccharide composed of glucosamine and hexuronic acid disaccharide repeating units (1). HS covalently attaches to core proteins to form HS proteoglycans (HSPG). Dictated by the location of the core proteins, HS chains present on cell surfaces, such as linking to syndecans and glypicans, and in the basement membrane by attaching to perlecans and agrins (1–3). HS biosynthesis is initiated by heterodimers formed by copolymerases Exostosin-1 (Ext1) and Exostosin-2 (Ext2) that elongate HS chains by alternatively adding glucuronic acid (GlcA) and N-acetylgalactosamine (GlcNAc) residues from their respective UDP-sugar nucleotide precursors. N-deacytelyase/N-sulfotransferase (Ndst) initiates modification reactions that occur on newly assembled HS chains, including N-, 3-O, and 6-O sulfation of GlcNAc units (NS, 3S, 6S, respectively), epitimerization of GlcA to iduronic acid (IdoA), and 2-O-sulfation of IdoA (2S). These modification reactions are incomplete, resulting in enormous structural diversity in mature HS and form a variety of ligand-binding sites to interact with a large number of protein ligands (1–3). The protein ligand-binding sites in HS often consist of relatively small tracts of variably sulfated glucosamine and uronic acid residues. For example, the antithrombin-binding site is composed of a specific pentasaccharide sequence: GlcNAc/NS(6S)-GlcA-GlcNS(6S)-GlcNS(3S6S)-IdoA(2S)-GlcNS(6S) (4). The FGF2 binding site is a short sulfated sequence with N- and 2-O-sulfation (5). Intriguingly, the generation of the ligand-binding sites is cell/tissue- and developmentally stage-specific, implying that the regulatory functions of HS occur in a temporal and spatial manner (6, 7).

Endothelial cells are one of the major cellular components of blood vessels that form the inner monolayer endothelium of blood vessels. Under normal physiological conditions, endothelial cells maintain vascular homeostasis and respond to
environmental changes to regulate inflammatory and immune response, vascular tone, coagulation, and fibrinolysis (8). Endothelial cells are also key players in angiogenesis that is finely tuned by the balance between pro- and anti-angiogenic factors (9). Angiogenesis plays an essential role in physiological conditions such as embryonic development, menstruation and wound healing, as well as in pathological conditions such as tumor growth, inflammatory disorders, eye diseases, stroke, etc (10). Therefore, understanding the mechanisms that control endothelial cell functions will greatly advance the development of effective treatment for vascular related human diseases.

HS is expressed abundantly in the vasculature. Genetic studies have established that HS is essential for endothelial cell function under physiological and pathological conditions (11). We previously reported that endothelial-specific knock-out of Ndst1 attenuates leukocyte adhesion and extravasation in inflammation (12), revealing that endothelial HS is required to facilitate leukocyte trafficking. This observation was confirmed by examining the same mutant mice by others (13, 14) and by endothelial-specific ablation of Ext1 (15) or HS 2-O-sulfotranserase (16). We also observed that deficiency of endothelial Ndst1 retards tumorigenesis (11), showing that endothelial HS critically promotes tumor angiogenesis. Recent studies by others also revealed that endothelial HS functions to promote vascular permeability (17) and tumor metastasis to lymph nodes (18). These studies illustrate HS to be a multifunctional molecule in vivo. To understand the underlying molecular mechanisms of HS on aforementioned endothelial cell functions, we examined the interactions of endothelial HS with one or several HS-binding ligands known to be involved in the studied biological functions. For example, we examined the interactions of endothelial HS with L-selectin and three chemokines involved in leukocyte trafficking (12), and the VEGF signaling in tumor angiogenesis (11). Similarly, only VEGF and neuropilin signaling were examined for their regulatory role of endothelial HS in vascular permeability (17), and only two chemokines were checked in the study of tumor metastasis to lymph nodes (18). This specific molecule targeting approach allowed us to understand some key underlying molecular mechanisms. However, in reality, a large number of HS-binding growth factors, morphogens, cytokines, and adhesion molecules are expected to co-exist at the examined conditions and HS may selectively interact with a fraction of these HS-binding protein ligands to modulate endothelial functions (3, 19). To fully understand these molecular mechanisms, an unbiased analysis is essentially needed to determine the cell-specific and spatiotemporal regulatory roles of HS on endothelial cell function and related signaling.

Regulatory post-translational modification represents a major cellular signaling mechanism that allows cells to sense environmental change. Among them, reversible phosphorylation controlled by kinases and phosphatases plays a central role in signaling in cell-cell or cell-environment communication (20). Global phosphoproteomics examines phosphorylation changes, and determines signaling regulation at a systemic level (21, 22). In this study, we applied the stable isotope labeling by amino acids (SILAC)1-based global quantitative phosphoproteomics analysis to examine HS-dependent endothelial signaling by comparing wild type and HS-deficient endothelial cells that were cultured in serum-containing medium. A total of 7222 phosphopeptides corresponding to 1179 proteins were identified. Bioinformatic analysis of the identified proteins determined that HS deficiency altered cellular functions that are highly related to cell morphology, cellular assembly and organization, cellular function and maintenance, inflammatory response and disorder, cell growth and proliferation, cellular survival and death, consistent with cell function studies, which found that HS deficiency alters endothelial cell growth and mobility. Mining for the underlying molecular mechanisms revealed that HS modulates signaling pathways critically related to cell adhesion, migration, and coagulation, and found that HS critically modulates IGF-1 signaling. In-depth analysis of growth factor signaling identified 22 HS-dependent growth factor/cytokine/growth hormone signaling factors, including both previously known and unknown ones such as IGF-1, erythropoietin, angiopoietin/Tie, IL-17A, and growth hormones. Twelve of the identified 22 HS-dependent signaling factors were alternatively confirmed by phospho-receptor tyrosine kinase (p-RTK) array analysis. Therefore, our results revealed a broad and very complex regulatory role of HS on endothelial signaling.

EXPERIMENTAL PROCEDURES

Mouse and Cell Line—The experimental mice harboring conditionally targeted Ext1 (Ext1f/f) mice were bred onto C57BL/6J background and housed at a specific pathogen-free facility as reported previously (23, 24). The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Georgia. Mouse lung endothelial cell (MLEC) lines were derived from 8-week-old Ext1f/f mice as described previously (12, 25). Briefly, the lungs were minced, digested at 37 °C for 1 h in Delbecco’s modified Eagles medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and various enzymes including collagenase Type II (200 U/ml, Invitrogen, 1 The abbreviations used are: BCA, bicinchoninic acid; DTT, dithiothreitol; DMEM, Dulbecco’s Modified Eagle Medium; Ext, Extosin; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FDR, false discovery rate; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; Glic, glucuronic acid; GlicNAc, N-acetylgalactosamine; GO, gene ontology; HGF, hepatocyte growth factor; HS, heparan sulfate; IGF-1, insulin-like growth factor 1; IGF-1R, insulin growth factor-1 receptor; IGFBP, insulin-like growth factor binding protein; LC, liquid chromatography; MLEC, mouse lung endothelia cells; Ndst, N-deacetylase/N-sulfotransferase; PDGF, platelet-derived growth factor; PE-cy5, R-Phycoerythrin-Cyanine dye 5; RTK, receptor tyrosine kinase; p-RTK, Phospho-receptor tyrosine kinase; SCX, strong cation exchange chromatography; SDF-1, stromal cell-derived factor 1; TFA, trifluoroacetic acid; SILAC, stable isotope labeling by amino acids; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
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Grand Island, NY), dispase (0.1%, Invitrogen), and DNase I (10 U/ml, MP Biosciences), and filtered through a 40 μm nylon strainer. The cell suspension was added with PE-conjugated rat anti-mouse CD31 (0.2 μg/10^6 cells, Invitrogen) and then incubated with magnetic beads coated with goat anti-PE antibody (20 μl/10^6 cells, Miltenyi Biotec Inc., Auburn, CA). Cells bound to the beads were recovered with a magnetic separator, expanded in culture, and immortalized by transfecting the primary cells with SV40 large T-antigen (26). The single colony of immortalized MLEC was picked out, expanded, authenticated by staining with antibodies specific to endothelial cell markers CD31 and vascular endothelial growth factor receptor-2 (VEGFR2), and frozen for future use. Ext1f/f→→ cell lines were generated by transient transfection of the Ext1f/f cell lines with a Cre-expressing plasmid followed by single cell cloning. Genotyping of the Ext1f/f and Ext1f/f→→ cells was performed as reported previously (23, 24, 27).

Flow Cytometry Analysis (25, 28)—Confluent MLECs in culture were detached with PBS containing 2 mM EDTA and 1% bovine serum albumin (BSA) (PBS-EB) and stained on ice for 30 min with FITC-conjugated-anti-CD31 (1:500, Invitrogen), PE-conjugated anti-VEGFR2 (1:500, BD Biosciences, San Jose, CA), anti-HS antibody (10E4 (1:50, Seigagaku, Tokyo, Japan), or their corresponding isotype control antibodies. After washing, the cells were analyzed by flow cytometry (Cell Lab QuantaTM SC, Beckman Coulter). To determine cell surface binding of protein ligands, the experiments were similarly carried out by first incubation with recombinant HGF or IGF-1 (2.5 μg/ml, both from Peprotech, Rocky Hill, NJ) and by second incubation with their corresponding biotinylated-antibodies (5 μg/ml, anti-HGF antibody, R&D Systems, Minneapolis, MN; anti-IGF-1 antibody, Peprotech) followed by PE-Cy5-streptavidin (1:500, BD Biosciences). For IGFBP3 binding, biotinylated-human IGFBP3 or BSA (1.25 μg/ml, Eagle Biosciences Inc. Nashua, NH) was incubated with cells at 4 °C for 30 min followed by incubation with PE-Cy5-streptavidin (1:500). Twelve thousand cell events were collected and the data was analyzed using the FlowJo software (Tree Star Inc., Ashland, OR).

Cell Proliferation Assay (28, 29)—Ext1f/f or Ext1f/f→→ MLECs (3000 cells/well) were seeded into a 96-well plate. Cell growth was assessed by colorimetric assay using the Cell Counting Kit-8 kit (Dojindo Molecular Technologies, Santa Clara, CA). The optical absorbance at 450 nm was read at 24-, 48-, and 72 h of culture using an OPTimax microplate reader (Tecan Group Ltd.).

Trans-well Migration Assay (28-30)—MLECs (5 × 10^4/well) were seeded into 8 μm transwell inserts (BD Bioscience). The low chamber was loaded with DMEM supplemented with 5% FBS. After culturing for 3 h, the cells on the filter were fixed in 90% ethanol for 10 mins. Nonmigrated cells on the upper surface of the filter were removed by gentle scraping with a cotton swab. Migrated cells on the lower surface of the filter were stained with 0.1% crystal violet and washed with distilled water until the water was colorless. Images of migrated cells were captured under an inverted microscope (Eclipse TE2000-S, Nikon) equipped with a Retiga 1300i FAST 1394 CCD camera (Olympaging Corp., Surrey, BC, Canada). The migrated cells were further quantified by extraction with 10% acetic acid and measured at 595 nm.

Wound Healing Recovery Assay (28, 29)—MLECs (2 × 10^5 cells per well) were seeded into a six-well plate and reached confluency after culture for 24 h. A wound gap was made by scratching with a T-200 tip in each well. After removing debris by PBS washing, the cells were cultured in DMEM medium supplemented with 10% FBS. Photos were taken immediately after the wounding or 72 h later under a SMZ1500 dissecting microscope (Nikon) at 3× magnification. The gap width of the wound was measured by Image J software (downloaded from http://rsb.info.nih.gov/ij/). The recovery ratio was calculated as [(Initial gap distance - current gap distance)/Initial gap distance] × 100%.

Tube Formation (28, 29)—MLECs (3 × 10^4/well) in DMEM medium containing 5% FBS were seeded on 96-well plate coated with growth factor reduced matrigel. After 6 h, cell morphology in culture was photographed under the Nikon Eclipse TE2000-S inverted microscope.

Phospho-Receptor Tyrosine Kinase (p-RTK) Array Assay—Mouse p-RTK Array (R&D Systems) was employed to determine the relative level of tyrosine phosphorylation of 39 different receptor tyrosine kinases (RTK) in Ext1f/f and Ext1f/f→→ MLECs cultured under normal serum-containing conditions, and the experiment was carried out according to the instructions provided by the manufacturer and as previously described (31). In brief, MLECs cultured in DMEM with 10% FBS was extracted with RIPA buffer (Pierce, Thermo Fisher) supplemented with phosphatase inhibitors (1:100, Sigma-Aldrich, St. Louis, MO) and protease inhibitors (1:10, Sigma-Aldrich). The sample supernatant was collected after centrifugation and quantified for protein concentration using a BCA kit. The mouse p-RTK array analysis was initiated by blocking the test membranes with Array Buffer 1 for 1 h and then incubating the membrane overnight at 4 °C with 500 μg sample protein prepared from Ext1f/f or Ext1f/f→→ MLECs. Following, the membrane was washed and incubated with a HRP-conjugated pan antiphospho-tyrosine antibody (1:5000, Cell Signaling Technology, MA). The binding of the antiphospho-tyrosine antibody was detected by chemiluminescence. The density of each blot was quantitated by Image J protein array analyzer macros (downloaded from http://rsb.info.nih.gov/ij/ and http://image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-ImageJ.html).

Western Blotting—Ext1f/f or Ext1f/f→→ MLECs were seeded into 6-well plate and incubated overnight. Cells were washed by PBS twice and extracted by adding 100 μl 1× SDS loading buffer (Bio-Rad Laboratories Inc.) to each well. The protein sample was denatured at 95 °C for 10 min and centrifuged at maximum speed for 10 min to remove insoluble materials. The supernatant was loaded onto SDS-PAGE gel and separated. The protein in the gel was transferred to nitrocellulose membrane (Bio-Rad Laboratories Inc.) by a semidy transfering system (Bio-Rad Laboratories Inc.) in constant voltage mode. The membrane was blocked by 5% nonfat milk for 1 h at RT. Following, primary antibody (1:500) for Met or IGF1R β (Cell Signaling Technology) was added and incubated overnight at 4 °C. β-actin served as an internal control. After further incubation with corresponding HRP-conjugated secondary antibody, the protein in the membrane was imaged by chemiluminescence.

Cell Labeling and Protein Extraction—Ext1f/f→→ MLECs (Heavy) were labeled with 13C6-lysine HCL and 13C6-arginine HCl (both from Cambridge Isotope Laboratories, Inc.) for at least five passages in medium supplemented with 10% dialyzed FBS (components with MW < 10 kDa were removed, Cat. no. S12650, Atlanta Biologicals, Lawrenceville, GA) (32). To extract cellular proteins, the cells were washed twice with cold PBS and then lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA) supplemented with protease and phosphatase inhibitor mixture (Roche, Branford, Connecticut). The supernatants were collected after the lysates were centrifuged at 17,000 × g for 15 min and protein concentration was quantitated using a BCA kit (Pierce). The same amount of Ext1f/f (Light) and Ext1f/f→→ (Heavy) protein samples were combined. The resultant mixture was added with four volumes of ice-cold acetone and precipitated overnight at ~−20 °C. The acetone precipitate was collected after centrifugation and reconstituted at 10 mg/ml in 50 mM ammonium bicarbonate containing 8 μl urea.

Protein Reduction, Alkylation, and Digestion—The reconstituted protein samples were reduced at RT for 45 min by adding 1 mM dithiothreitol (DTT) to a final concentration 1 mM with shaking. The sample was further subjected to alklylation in the dark for 30 min after...
adding iodoacetamide to final concentration at 5.5 mM, diluted with four volumes of 50 mM ammonium bicarbonate to achieve a final concentration of urea below 2 M, and digested overnight at 37 °C with sequence grade trypsin (20 ng enzyme per μg lysates, Promega, Madison, WI). The digestion was terminated by the addition of trifluoroacetic acid (TFA). The resulting peptide mixtures were desalted with a Sepak C18 cartridge (Waters, Milford, MA) and lyophilized.

SCX (Strong Cation Exchange Chromatography) Fraction—The lyophilized peptide mixtures were fractioned with an Agilent 1200 HPLC system as reported (33). Briefly, the peptide mixture (6–10 mg) was resolved in Buffer A (30% acetonitrile, 7 mM KH2PO4, pH 2.65 after adjustment with TFA) and loaded onto a Resource 15S 4.6/100PE (GE Health Science, Pittsburgh, PA) column that was pre-equilibrated with Buffer A. The loaded peptide sample was eluted out using a linear gradient of KCl ranging from 0 - 350 mM by mixing Buffer A with Buffer B (Buffer A + 350 mM KCl) at a flow rate of 1 ml/min. The eluted samples were collected for 4 min per fraction. Following, the collected fractions were combined into 10 fractions and lyophilized.

Phosphopeptide Enrichment—Each fraction of the lyophilized peptides was desalted using a Sepak C18 Cartridge (Waters) with Buffer C (0.4% TFA/60% Acetonitrile solution, 25% lactic acid) as the elution buffer. Phosphopeptide was enriched using a TiO2 column (TitanSphere PHOS-TiO Kit, GL Sciences, Torrance, CA) (34). Briefly, the TiO2 column was sequentially equilibrated with Buffer D (0.4% TFA/60% Acetonitrile) and Buffer C. Following, the sample in Buffer C was loaded to the column and sequentially washed with Buffer D once and then Buffer C twice. The bound phosphopeptides were eluted out with 5% ammonium hydroxide solution followed by 5% pyridine solution.

LC-MS/MS Analysis—Phosphopeptides were analyzed using the Eksigent nanoLC-UltraTM2D System combined with the cHiPLCTM-Nanoflex system in Trap-Elute mode. The peptides were loaded on the cHiPLC trap (200 μm x 500 μm ChromXP C18-CL 3 μm 300 Å) and washed for 10 min at 2 μl/min. An elution gradient of 5–35% acetonitrile (0.1% formic acid) over 110 min at 300 nL/min was then applied on a nano cHiPLC column (75 μm x 15 cm ChromXP C18-CL 3 μm 300 Å). Surveys of full scan MS spectra (from m/z 375 to 1700) were acquired in the AB SCIEX TripleTOF® 5600 System with a resolution of 40,000 for both MS and MS/MS. The 50 most intense ions (depending on signal intensity) were sequentially isolated for fragmentation in the quadrupole by collision-induced dissociation. The interface temperature was maintained at 150°C and the spray voltage was kept at 2500 V.

Data Analysis—All raw data was processed using ProteinPilot™ Software 4.5 (AB Sciex, Framingham, MA) with integrated false discovery rate analysis. The output files were searched against the International Protein Index mouse protein sequence database (version 3.87 with 535,248 entries) using the Paragon search algorithm 4.5.0.0, 1654 (35) with common contaminants included. Search parameters are set as sample type [SILAC (Lys+=6, Arg+=6)], cys alkylation (iodoacetamide), digestion (Trypsin), instrument (TripleTOF 5600), special factors (phosphorylation emphasis and urea denaturation), species (Mus musculus), ID Focus (Biological modifications), database (uniprot_sprot.fasta), search effort (Thorough), false discovery rate (FDR) analysis (Yes), and user modified parameter files (No). The proteins were inferred based on the ProGroup™ algorithm associated with the ProteinPilot software. The tolerance of precursor ion and product ion was set at 0.05 Da and 0.05 Da, respectively. The detected protein threshold (unused ProtScore) was set to 1.3 (95% confidence). Quantitation was based on peak area of each peptide identified. Quantitative phosphopeptide selection criteria are as following: 1). The phosphopeptides without quantitative information were discarded. 2). The phosphopeptides that were annotated with “auto-discordant peptide type” and “auto-shared MS/MS” were excluded. All peptides and proteins were filtered with confidence to 5% FDR. The count of phosphorylation sites such as phospho-serine (p-Ser), phospho-threonine (p-Thr), and phospho-tyrosine (p-Tyr) in the data set was calculated using in-house perl-based scripts with minor modification (36). Peptides were defined as redundant if they had identical cleavage site(s), amino acid sequence, and modification. A score of each phosphorylation site was calculated by in-house developed python scripts to evaluate the phosphorylation site confidence. Phosphosite with A-score >10 was considered to be near certainty (37).

Bioinformatics—Gene ontology (GO) annotation of the identified proteins was obtained using the software DAVID (38). All quantified phosphopeptides were analyzed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) by core analysis. The over-represented biological processes, molecular functions, and canonical pathways were generated based on information contained in the Ingenuity Pathways Knowledge Base. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Fisher’s exact test was used to calculate a p value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

RESULTS

Experimental Strategy—To unambiguously determine the regulatory role of HS on endothelial cell function and related signaling, we initially tried to obtain endothelial cells that are deficient in HS expression. For this purpose, we targeted HS biosynthetic gene Ext1 to generate Ext1 null (Ext1 −/−) MLEC lines with proper wild type (Ext1+/+) control (Fig. 1A). Second, the SILAC-based quantitative phosphoproteomic analysis was carried out to determine phosphorylation alterations that were caused by Ext1 ablation in MLECs (Fig. 1B).

Derivation of Ext1+/+ and Ext1+/− MLEC lines—To derive Ext1+/− MLEC lines, the “mother” Ext1+/+ MLEC lines were initially generated by immortalizing primary MLECs in culture and the following cell cloning (Fig. 1A). Second, the Ext1+/+ MLEC lines were transiently transfected with Cre-recombinase followed by single cell cloning and PCR determination of Ext1 ablation to screen for “daughter” Ext1−/− MLECs (Fig. 2A). The enzyme Ext1 functions to initiate HS biosynthesis by polymerizing the HS precursor structure. We and others reported previously that inactivation of Ext1 completely disrupts HS expression in mouse embryonic stem cells (23, 24, 39). To determine whether Ext1 ablation similarly diminishes HS expression in the derived MLEC lines, the Ext1+/+ and Ext1−/− cells were stained with an anti-HS antibody (10E4) and analyzed by flow cytometry. Ext1+/+ cells express abundant cell surface HS corresponding to a much higher fluorescence signal of 10E4 staining compared with isotype-matched control staining (Fig. 2B). In contrast, there is nearly no difference in the Ext1−/− cells between 10E4 and the isotype-matched control staining (Fig. 2C), showing directly that Ext1 ablation completely disrupts HS biosynthesis in the Ext1+/− MLECs. To confirm that the derived cells are true endothelial cells, the cells were stained with antibodies specific to CD31.
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Fig. 1. Experimental strategy. A, Derivation of Ext1f/f and Ext1–/– MLEC lines. Primary MLECs were isolated from Ext1f/f mice and immortalized to obtain Ext1f/f (wildtype) MLEC lines through cell cloning. The Ext1f/f MLEC lines were then transfected with Cre-recombinase to ablate the floxed Ext1 allele to generate Ext1 deficient (Ext1–/–) “daughter” MLEC lines. The derived cells were further examined for basic endothelial cell functions. B, Phosphoproteomics analysis. Light, unlabeled protein sample from Ext1f/f MLECs; Heavy, 13C6-L-lysine HCl labeled protein sample from Ext1–/– MLECs; Enriched Phosphopeptides.

Fig. 2. Characterization of the derived Ext1f/f and Ext1–/– MLEC lines. A, PCR analysis of genomic DNA isolated from the Ext1f/f and Ext1–/– MLEC clones. Ext1f: floxed Ext1 allele (wildtype); Ext1–: mutant Ext1 allele. B–G, Flow cytometry analyses of cell surface expression of HS (B, C), and of endothelial cell markers CD31 (D, E) and VEGFR2 (F, G) by staining with corresponding antibodies (blue peaks) or isotype background controls (red peaks).

or VEGFR2, the two common endothelial cell markers. Both the Ext1f/f and Ext1–/– cells express high levels of CD31 and VEGFR2 on their cell surface (Fig. 2D–2G), ensuring that both cell lines are indeed endothelial cells. Furthermore, the Ext1f/f and Ext1–/– cells both exhibit cobblestone-like morphology in culture (data not shown), which is typical for endothelial cells and morphologically supports that the derived cell lines are endothelial cells. Taken together, using conditional gene targeting and cell immortalization approaches, we successfully derived HS deficient (Ext1–/–) MLECs and their wild type (Ext1f/f) control MLECs. Because the “daughter” Ext1–/– cells were derived from the “mother” Ext1f/f control MLECs, both cell lines have the same genetic background. Therefore, the Ext1f/f and Ext1–/– MLECs provide a genetic background and fully matched experimental system allowing us to vigorously determine the regulatory role of HS on endothelial cell function and related signaling.

HS Regulates Endothelial Cell Functions—To determine whether HS essentially modulates cell functions of the derived MLECs under physiological condition (Fig. 1A), we initially examined the proliferation rate of the Ext1f/f and Ext1–/– MLECs cultured in FBS-containing DMEM, the medium routinely used in the cell culture. At 10% FBS containing condition, the Ext1–/– MLECs appeared to grow slower compared with Ext1f/f cells, but the difference was not statistically significant (Fig. S1). Whereas, when FBS concentration was reduced to 1%, the growth retardation of Ext1–/– MLECs became highly significant (Fig. 3A). Furthermore, the growth
Retardation could be partially rescued by supplementing the culture with heparin, a highly sulfated form of HS (Fig. 3A). These observations demonstrate that HS essentially promotes endothelial cell growth.

High mobility is critically required for some endothelial cell-mediated biological processes, such as vessel sprouting to form new vasculature during angiogenesis. Therefore, we further determined whether HS modulates mobility of endothelial cells. Cell motility includes two types of cell movement, the autonomous migration and chemotaxis. We initially determined whether Ext1 ablation affected autonomous migration of MLECs by employing the wound healing recovery assay. The migration of MLECs from the wounded edge into the denudated area was photographed and quantified by measuring the gap width. As shown in Fig. 3B–3C, compared with the Ext1+/f cells, Ext1−/− cells were less competent in repair of the wounded area. Quantification revealed that 72 h after the wounding, the Ext1+/f MLECs recovered 76% of the gap. In contrast, Ext1−/− MLECs only recovered 16%. To determine whether HS modulates chemotaxis of endothelial cells, we carried out the Boyden Chamber trans-well migration assay by supplementing 5% FBS containing DMEM in the lower chamber with MLECs loaded in the upper chamber containing DMEM only. The MLECs that migrated onto the bottom side of the trans-well membrane were photographed and quantified. Compared with Ext1+/f cells, significantly less Ext1−/− MLECs migrated across the trans-well membrane (Fig. 3D–3E). Parallel experiments showed that supplementing heparin in culture was close to fully rescuing the defects of wound healing and trans-well migration of the Ext1−/− MLECs, confirming that the cell mobility defects are due to HS deficiency (Fig. 3B–3E). Together, these observations demonstrated that...
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HS potently enhances both autonomous migration and chemotaxis to promote endothelial cell mobility. 

One unique functional feature of endothelial cells is their capability to form capillary-like structures on matrigel, a multistep vascular morphogenesis process involving cell adhesion, migration and growth. To assess whether HS modulates this complex vascular morphogenesis process, Ext1f/f and Ext1−/− MLECs were seeded and cultured on matrigel-coated wells. At this culture condition, Ext1f/f MLECs formed long and well-linked tubule-like structures within 6 h, whereas in sharp contrast, the Ext1−/− cells remained individually or as a few cells clustered together forming short and disconnected tubular structures, and the tube formation defect could be potentially rescued by heparin supplement in culture (Fig. 3F–G). Consistent with this observation, under normal culture condition without matrigel, the Ext1f/f MLECs formed tubular structures after reaching confluence, whereas, the Ext1−/− MLECs remained at all times as a sheet-like monolayer (supplemental Fig. S2). These observations are in agreement with the afore-observed pro-migratory role of HS on endothelial cell mobility. Together, these observations show that HS critically modulates endothelial cell functions and also demonstrate that the Ext1f/f and Ext1−/− MLECs are a new cell model system to use to investigate the role of HS in endothelial signaling.

Quantitative Phosphoproteomic Profiling of Ext1f/f and Ext1−/− MLECs—To systematically analyze the regulatory role of HS in endothelial signaling, we employed SILAC-based quantitative phosphoproteomic analysis. Phosphopeptides were prepared and analyzed in triplicate as detailed in the Experimental Procedures section. Phosphopeptides with minimum confidence of 90%, which were determined by ProteinPilot, were accepted according to a previous report (40). We cannot set missed and/or nonspecific cleavages permitted parameter in the ProteinPilotTM software for the triple TOF5600 data. From the results, we can see at least two missed and/or nonspecific cleavages. A total of 7222 unique phosphopeptides, which correspond to 1179 proteins, were identified in both Ext1f/f and Ext1−/− MLECs (supplemental Table S1). Among them, there were 2831 distinct peptides corresponding to the 884 proteins quantified using the ProteinPilot Software (supplemental Table S2). Among the 7222 identified phosphopeptides, 6892 phosphopeptides contained phospho-Ser, 803 phosphopeptides contained phospho-Thr, and 29 phosphopeptides contained phospho-Tyr site. The ratio of phospho-Ser to phospho-Thr to phospho-Tyr site was 237:28:1 and agrees with the literature (41). Analysis of the phosphosite number in the identified phosphopeptides revealed that most of the phosphopeptides are singly phosphorylated (92.55%), and 7.26% of the phosphopeptides are doubly phosphorylated. A few phosphopeptides were identified with three or more phosphorylation sites and were discarded after manual inspection of the corresponding MS/MS spectra. The raw data has been provided in a public database PeptideAtlas and can be accessed through the following link: https://db.systemsbiology.net/sbeams/cgi/Pep tideAtlas/PASS_View?identifier = PASS00235.

Functional Distribution of Identified Phosphoproteins—To understand the biological functions of the proteins identified, we performed GO analysis of the identified phosphoproteins. There are three main GO categories, namely, cellular component, molecular function, and biological process. The GO enrichment was accomplished using the software DAVID and revealed that most of the identified proteins are associated with organelles, cytoskeleton, plasma membrane, and cytosol (Fig. 4A). The most relevant biological processes identified include transcription control, protein location and translation, intracellular signaling cascade, phosphorylation and phosphorelay metabolism, cell cycle, cell death, and transportation (Fig. 4B). Consistent with these findings, the GO analysis of molecular functions further revealed that the identified proteins were tightly related to the binding of various nucleotides, kinase activity, and transcription regulator activity (Fig. 4C).

Functional Signaling Network Modulated by HS—To decipher endothelial signaling regulated by HS, we performed core analysis of the quantified phosphopeptides using the Ingenuity Pathway Analysis (IPA) software. In total, 25 functional networks were identified to be HS dependent. The top five identified functional networks include cell morphology, cellular assembly and organization, cellular function and maintenance, inflammatory response and disorder, cell growth and proliferation, cellular survival and death (Table I). In addition to the specific networks, multiple canonical signaling pathways that have been known to be modulated by HS were also identified, such as VEGF and FGF signaling, thus verifying the credibility of the data analysis of the IPA software (supplemental Fig. S3). Surprisingly, the top HS-dependent signaling identified is IGF-1 signaling, which previously had not been known to be HS-dependent. In addition, adhesion signaling was also identified in the top-ranked HS-dependent signaling, including FAK signaling and integrin signaling (supplemental Fig. S3). Meanwhile, HS was identified to modulate other signaling critically related to endothelial cell mobility, including actin cytoskeleton organization and cell junction (supplemental Fig. S3). Furthermore, the thrombin signaling, a key process in blood coagulation, was observed to be altered (supplemental Fig. S3), indicating that HS may critically modulate hemostatic competency of endothelial cells.

HS-modulated Growth Factor Signaling—Several growth factors, such as VEGF, essentially modulate the basic cellular functions of endothelial cells as afore-examined in this study. Therefore, specific attention was paid to examine growth factor signaling modulated by HS. The IPA analysis determined that, compared with Ext1f/f MLECs, 22 growth factor/cytokine/growth hormone signaling pathways were altered in the Ext1−/− cells, including the well-documented HS-dependent signaling such as VEGF, FGF, PDGF, TGFβ, HGF,
GM-CSF, and neuregulin/ErbB (Fig. 5A). This analysis also revealed several signals that have not been known to be HS-dependent, including IGF-1, erythropoietin, angiopoietin/Tie, IL-17A, and growth hormones (Fig. 5A). To alternatively confirm these findings, we carried out mouse p-RTK array analysis. The p-RTK array examined 39 different phosphorylated mouse RTKs and included 12 signaling pathways that were identified in our phosphoproteomics analysis to be HS-dependent (Fig. 5A, supplemental Fig. S3 and Table II). All of the 12 growth factor signalings, that were altered in Ext1−/− MLECs in phosphoproteomics analysis, were down-regulated in the p-RTK array analysis (Figs. 5B–5C and Table II). Several growth factor signalings, such as Mer, that were unaltered in the phosphoproteomic analysis remained no different between Ext1f/f and Ext1−/− MLECs in the p-RTK kinase array assay (Figs. 5B–5C, Table II), alternatively showing that the

Fig. 4. GO analysis of the functional distribution of the identified phosphoproteins by DAVID. A, Cellular component. B, Biological process. C, Molecular function.
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| ID | Associated Network Functions                                      | Score |
|----|------------------------------------------------------------------|-------|
| 1  | Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance | 32    |
| 2  | Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance | 32    |
| 3  | Inflammatory Disease, Inflammatory Response, Ophthalmic Disease   | 30    |
| 4  | Cell Cycle, Cellular Growth and Proliferation, Cellular Movement  | 25    |
| 5  | Cell Death and Survival, Cancer, and Hematological Disease        | 25    |

Phosphoproteomic analysis is sensitive, specific, and accurate to determine HS-dependent growth factor signaling in endothelial cells.

Cell surface HS has been proposed to function as a coreceptor to facilitate a large variety of growth factor signaling. To validate our findings in phosphoproteomics and p-RTK array analyses and to test whether the newly identified HS-dependent growth factor signaling follows a similar coreceptor model, we used flow cytometry analysis to examine whether HS deficiency could affect cell surface binding of HGF and IGF-1. As shown in Fig. 6A–6B, Ext1f/f MLECs exhibited abundant HGF cell surface binding, whereas the HGF binding to Ext1−/− cells is greatly diminished although the mutant cell expresses normal levels of the HGF receptor (HGFR). This shows that HS deficiency impairs HGF binding to the HGF receptor on the cell surface and leads to the disruption of HGF signaling in Ext1−/− MLECs. Because HGF is known to bind HS (42), this observation also indicates that HS indeed acts as a coreceptor to facilitate HGF signaling. IGF-1 functions to bind and phosphorylate IGF receptor-1 (IGF1R) and the insulin receptor to initiate IGF-1 signaling. FACS analysis demonstrated that the binding of IGF-1 to Ext1−/− MLECs is also diminished (Fig. 6C). However, IGF-1 is a non-HS-binding protein (43) and Ext1 ablation does not alter IGF1R expression (Fig. 6B), therefore the diminished cell surface IGF-1 binding indicates that cell surface HS interacts with other molecules to facilitate IGF-1 signaling. To test this idea, we further examined whether the Ext1 ablation affects cell surface binding of the IGF-binding protein (IGFBP). IGFBPs are 6-member protein family and have high affinity to IGF-1. IGFBPs function through three mechanisms to modulate IGF signaling, including IGF transportation, protection of IGF from degradation, and regulating the interaction between IGF and IGF1R (44). IGFBP2–6 is secreted by endothelial cells and bind on the endothelial cell surface (45). Interestingly, IGFBPs bind heparin, especially IGFBP3 and IGFBP5, both show high affinity binding to heparin (45). Furthermore, heparin, IGF-1, and IGFBP have been observed to form ternary complexes transiently (43), suggesting that endothelial cell surface HS may function in a similar way to modulate IGF-1 signaling. To test this idea, cell surface IGFBP3 binding was examined by flow cytometry analysis. The Ext−/− MLECs showed significantly reduced cell surface IGFBP3 binding (Fig. 6D), suggesting that endothelial cell surface HS functions through binding of IGFBPs to facilitate IGF-1 signaling.

**DISCUSSION**

Recent genetic studies showed that HS critically modulates endothelial cell functions, including mediation of leukocyte trafficking, inflammation, angiogenesis, vascular permeability, and tumor metastasis (19, 46). However, elucidation of their underlying cellular and molecular mechanism has been challenging. This is mainly because in tissues, endothelial cells reside in an environment with a large number of HS-binding ligands (3). All these presenting HS-binding proteins have the potential to modulate endothelial signaling in a HS-dependent manner, thereby regulating endothelial cell functions. Previous studies revealed the interactions of endothelial HS with a few selected protein ligands that are known to be critically involved in leukocyte trafficking (12), inflammation (15), tumorigenesis (11), vascular permeability (17), and tumor metastasis (18). These interaction studies have led us to partially understand the underlying cellular and molecular mechanisms. In this report, we employed SILAC-based quantitative phosphoproteomic analysis in combination with genetic ablation of HS expression to examine global HS-dependent biological functions and signaling in endothelial cells under normal FBS-containing culture conditions. Our results show that HS modulates 25 functional networks, and the top five are critically related to cell morphology, cellular assembly and organization, cellular function and maintenance, inflammatory response and disorder, cell growth and proliferation, cellular survival and death. Bioinformatic analysis for the underlying molecular mechanisms revealed that HS modulates pathways related to cell adhesion, migration, and coagulation, such as ILK signaling, integrin signaling, signaling related to actin cytoskeleton organization, and tight junction and thrombin signaling. Meanwhile, our molecular signaling analysis unexpectedly identified that the top HS-dependent pathway is IGF-1 signaling, uncovering that HS may critically modulate IGF-1 signaling-mediated cell functions. Our detailed analysis of growth factor signaling revealed 22 growth factor/cytokine/growth hormone signaling pathways that were modulated by HS, including several signaling pathways that have not been known to be HS-dependent, including IGF-1, erythropoietin, angiopoietin/Tie, IL-17A, and growth hormones. In summary, this SILAC-based phosphoproteomic analysis confirmed previous findings and also uncovered novel HS-dependent functional networks and signaling, thus advancing our understanding of the regulatory role of HS on endothelial function and the underlying molecular mechanisms from a systematic view.
In this study, many pathways were identified to be HS-dependent (supplemental Fig. S3), reflecting the complexity of the regulatory roles of HS on endothelial cell function and signaling at systematic levels. This general observation is consistent with the complex heterogeneous HS structural features that enables HS to interact with a large number of growth factors, growth factor binding proteins, extracellular proteases, protease inhibitors, chemokines, morphogens, and adhesive proteins. Sorting the identified signaling pathways revealed that the top ranked HS-dependent signaling pathways were IGF-1 signaling, integrin signaling, and signaling related to actin cytoskeleton organization and cell junction. These top ranked signaling pathways are critically related to cell proliferation, apoptosis, cell adhesion, and migration (supplemental Fig. S3), and are directly correlated with the cell status in culture where the MLEC are at the exponential growth phase undergoing active cell proliferation and migration. These observations lead us to reason that HS...
deficiency impairs cell proliferation, cell adhesion, and migration signaling, resulting in arrested cell growth, mobility, and tube formation of the Ext1−/− MLECs as observed in our cell function studies (Fig. 3).

Our studies are in agreement with a recent study reporting that HS and heparin, a highly sulfated form of HS, bind a large number of proteins to form a highly interconnected network, which is functionally linked to physiological and pathological processes (47). In this study, we identified 22 growth factor/cytokine/growth hormone signaling pathways that were regulated by HS, which included previously known growth factor signaling pathways such as VEGF, FGF, HGF, NGF, HB-EGF, PDGF, GM-CSF, and TGF-β (3), and unknown signaling pathways such as IGF-1, angiopoietin/Tie, erythropoietin, IL-17A and growth hormone signaling (Fig. 5). While mining the other signaling pathways listed in supplemental Fig. S3, we identified that endothelial HS also modulates the signaling of other soluble protein ligands, including chemokines SDF1-α, MCP-3 and IL-8, as well as the axon guidance molecule Netrin (supplemental Fig. S3). These findings demonstrated that HS simultaneously modulated the signaling of a large number of soluble effectors and the net effect exhibited by cellular functions should reflect the integration of these signals in the cells. Interestingly, in our study, all the identified signaling pathways were down-regulated in Ext1−/− MLECs, revealing that HS functions as a general “accelerator” of all major pathways, including the multiple receptor tyrosine kinase signaling pathway, to promote cell growth, adhesion, and migration. Recent studies emerged showing that simultaneous inhibition of receptor tyrosine kinases, especially the HGF and VEGF signaling, potently inhibited cell proliferation of drug resistant cancer cells, presenting a novel approach to potentially cure cancers (48–50). Our findings suggest that simultaneous inhibition of multiple receptor tyrosine kinases may be achieved by targeting HS only, inspiring a great po-
shown to be pro-angiogenic both in vitro and in vivo (53, 54), and endothelial-specific ablation of IGF1R was observed to arrest angiogenesis in skin (55), thus establishing the IGF-1/IGF pathway to critically promote angiogenesis. In this study, we unexpectedly observed that ablation of Ext1 down-regulates IGF-1 signaling, revealing a novel molecular mechanism by which endothelial HS functions to promote angiogenesis. Our studies also suggest that endothelial HS interacts with IGFBP to indirectly facilitate IGF-1 signaling. However, further detailed studies, such as examination of cell surface formation of the HS/IGFBP/IGF-1 ternary complex and its subsequent effect on downstream intracellular signaling, are clearly needed to fully understand how HS modulates IGF-1 signaling. These further studies may open a new direction to decipher the regulatory mechanism of HS in endothelial cell function and in aberrant IGF signaling-based diseases, such as diabetes.

To the best of our knowledge, this study may represent the first report investigating the regulatory role of HS on endothelial signaling at a systematic level among a few proteomics studies of endothelial cells (56). Limited to the difficulty in maintaining primary MLEC s long enough in culture to reach satisfied SILAC-based labeling and in obtaining Ext1+/−/− primary MLECs because of embryonic lethality of the endothelial-specific Ext1 knockout mice (Zhang SY et al. manuscript in preparation), in this study we used immortalized MLECs by generating wild-type “mother” Ext1+/+ and “daughter” HS-deficient Ext1−/− MLEC lines. The Ext1+/+ and Ext1−/− MLECs have the same genetic background, therefore providing a best matched model system to determine the regulatory role of HS on endothelial cell functions and the underlying signaling. Because our phosphoproteomic results are fully consistent with the known HS-dependent signaling mechanisms found in the literature and were reconfirmed by performing p-RTK array analysis in parallel, we are confident that our results are reliable and representative. Considering that the immortalized MLECs are still somewhat different from primary cells as well as that endothelial cells from different vascular beds/organisms have their own unique characteristics, we also expect that the systematic view of HS regulation on primary endothelial cell signaling and the signaling of endothelial cells from different vascular beds or organs might be somewhat different.

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This article contains supplemental Figs. S1 to S5 and Tables S1 and S2.

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