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Endothelial Heparan Sulfate in Angiogenesis

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Abstract

Heparan sulfate (HS) is a linear polysaccharide composed of 50–200 glucosamine and uronic acid (glucuronic acid or iduronic acid) disaccharide repeats with epimerization and various sulfation modifications. HS is covalently attached to core proteins to form HS-proteoglycans. Most of the functions of HS-proteoglycans are mediated by their HS moieties. The biosynthesis of HS is initiated by chain polymerization and is followed by stepwise modification reactions, including sulfation and epimerization. These modifications generate ligand-binding sites that modulate cell functions and activities of proteinases and/or proteinase inhibitors. HS is abundantly expressed in developing and mature vasculature, and understanding its roles in vascular biology and related human diseases is an area of intense investigation. In this chapter, we summarize the significant recent advances in our understanding of the roles of HS in developmental and pathological angiogenesis with a major focus on studies using transgenic as well as gene knockout/knockdown models in mice and zebrafish. These studies have revealed that HS critically regulates angiogenesis by playing a proangiogenic role, and this regulatory function critically depends on HS fine structure. The latter is responsible for facilitating cell-surface binding of various proangiogenic growth factors that in turn mediate endothelial growth signaling. In cancer, mouse studies have revealed important roles for endothelial cell-surface HS as well as matrix-associated HS, wherein signaling by multiple growth factors as well as matrix storage of growth factors may be regulated by HS. We also discuss important mediators that may fine-tune such regulation, such as heparanase and sulfatases; and models wherein targeting HS (or core protein) biosynthesis may affect tumor growth and vascularization. Finally, the importance of targeting HS in other human diseases wherein angiogenesis may play pathophysiologic (or even therapeutic) roles is considered.

I. Endothelial Heparan Sulfate in Developmental and Physiologic Angiogenesis

A. Angiogenesis in Health and Disease: An Introduction

Angiogenesis refers to the generation of new blood vessels from existing ones, a process that differs from vasculogenesis (the *de novo* formation of blood vessels from mesoderm or endothelial cells (EC) progenitors).^{1–3} Angiogenesis occurs through an orchestrated sequence of events consisting of two general processes: formation of a nascent vascular network and subsequent maturation. To form a nascent vascular network, ECs first sprout

from existing capillary plexus in response to angiogenic stimuli, degrade the extracellular matrix (ECM), and then migrate into avascular tissue, assembling into an endothelial network. The proliferation and migration of ECs are potently modulated by growth factors, such as vascular endothelial growth factor (VEGF). The maturation process includes stabilization of the newly formed vessel with the recruitment of mural cells (MC; including vascular smooth-muscle cells (VSMC) in arteries, arterioles, and veins; and pericytes in capillaries), remodeling (branching, pruning, and regression of vasculature), and vessel specialization that determines arterio-venous commitment as well as organ-specific networks.^{4–8} The maturation process is critically modulated by growth factors, including platelet-derived growth factor-B (PDGF-B) and transforming growth factor- β (TGF- β).

Physiological angiogenesis occurs mainly during embryonic development and in postnatal growth of tissues such as the retina.^{1,9} In adults, it takes place primarily in the female reproductive system and in the intestinal villa.^{10,11} However, abnormal angiogenesis plays a critical role in the pathogenesis of many diseases such as cancer, ischemic vascular disorders, and diabetic retinopathy.^{1,12} Tumor-associated neovascularization (tumor angiogenesis) is required not only for tumor growth, but also for metastasis. Therefore, blockage of tumor angiogenesis represents a promising approach for the development of new cancer therapeutics. Stimulation and promotion of a mature neovascular network, referred to as therapeutic angiogenesis, shows promise as a treatment for ischemic diseases such as ischemic coronary artery disease and stroke.^{1,8,12,13}

B. Heparan Sulfate in Development

Heparan sulfate proteoglycans (HSPGs) are glycoconjugates composed of a core protein with one or more covalently attached heparan sulfate (HS) chains (Fig. 1).^{14–16} HSPGs are present abundantly on the cell surface and in the ECM where they interact with numerous growth factors, growth factor binding proteins, extracellular proteases, protease inhibitors, chemokines, morphogens, and adhesive proteins.^{14–17} These complex interactions regulate the activity, gradient formation, and stability of many ligand–receptor interactions. Most of the interactions of HSPGs with the ligands are mediated directly by the HS moieties.^{14–16,18} HS is a linear polysaccharide composed of glucosamine and uronic acid (glucuronic acid, GlcA, or iduronic acid, IdoA) disaccharide repeats with various sulfation modifications (Fig. 1), and is typically 50–200 disaccharides in length. HS biosynthesis is initiated by copolymerases Ext-1 and Ext-2, which alternatively add GlcA and *N*-acetylglucosamine residues from their nucleotide sugar precursors (Fig. 1).^{15,16} Following chain elongation, *N*-deacetylase/*N*-sulfotransferases (*N*dsts) act on discrete regions of the HS precursor, replacing *N*-acetyl groups with *N*-sulfates.¹⁹ Regions of *N*-sulfation (NS-domain) then act as the substrate for additional modifications, including epimerization, and 2-O-, 6-O-, and 3-O-sulfation.^{15,20} The modifications are generally incomplete and the enzymes involved have substrate specificities, which result in IdoA and O-sulfate residues occurring predominantly in the NS-domains. The sulfation pattern within the NS-domains, as well as their length and spacing, contribute to the structural heterogeneity of HS and create specific binding sites for protein ligands (Fig. 1).^{21–23} Intriguingly, the distribution, length, and modification level of such domains appear to be tightly regulated in a tissue/cell-specific fashion,²⁴ suggesting that the regulatory role of HS occurs in a spatiotemporal manner by interacting with unique arrays of protein ligands in different tissues and at different developmental/pathological stages.^{24,25} Loss-of-functional studies of HS biosynthetic genes in mice proved this concept by observing phenotypes ranging from early developmental defects (*Ext-1*, *Ext-2*), prenatal/neonatal lethality (*Ndst-1*, 2-O-sulfotransferase (*Hs2st*), C5-epimerase (*Hepi*), 3-O-sulfotransferase 1 (*Hs3st-1*), 6-O-sulfo-transferase 1 (*Hs6st-1*)), to a restricted heparin biosynthesis defect (*Ndst2*) (Table I). Further studies to elucidate the distinct molecular pathways modulated by HS, the HS structure–function relationship, and the underlying

cellular and molecular mechanisms in the development of different organs remain as important tasks in the field.

C. Heparan Sulfate in Developmental Angiogenesis: Overview of Genetic Evidence

Blood vessels constitute the first organ in the embryo and form the largest network in the body. Therefore, the formation of new blood vessels accounts for one of the major events during embryonic development. Vascular formation is tightly controlled by the balance between pro- and antiangiogenic factors.¹ HS has been shown to facilitate both pro- and antiangiogenic factors *in vitro*.^{31,49–52} These observations have spurred interest in the general role of HS in angiogenesis *in vivo*. This has recently been examined through genetic manipulation of HS biosynthetic genes in vertebrate model organisms of zebrafish and mice.

1. Zebrafish Studies³⁴—HS has been shown to be an important modulator of patterning processes during organogenesis in development. RNA interference of the *Drosophila Hs6st* produces defective branching in the tracheal system.⁵³ Since *Drosophila* tracheogenesis and vertebrate vasculogenesis share many common molecules, such as FGFs, VEGF, and integrins, tracheogenesis in *Drosophila* is thought to be a close model for mammalian vascular development.⁵⁴ The tracheal branching defect observed in the *Hs6st* mutant *Drosophila* suggests that *Hs6st* may modulate vascular branching in vertebrates.⁵³ This was tested in zebrafish by Chen et al.³⁴ In zebrafish, *Hs6st* exists in two isoforms (*Hs6st-1* and *-2*). *In situ* hybridization reveals dynamic and distinct expression patterns of these two genes during development. *Hs6st-2* is expressed in the cells surrounding the dorsal aorta and posterior cardinal vein, suggesting that *Hs6st-2* might have a role in vascular development or function. Indeed, *Hs6st-2* morphants exhibit abnormalities in the vascular branching morphogenesis of the caudal vein during embryonic development, showing vascular dysmorphogenesis ranging from reduced branching accompanying formation of large loops to a lack of branching with disorganized and overlumenized vessels in place of the venous plexus. Although *Hs6st-1* has biochemical activity similar to *Hs6st-2*, vascular morphogenesis in *Hs6st-1* morpholino-injected embryos is normal, which is consistent with the lack of *Hs6st-1* expression in developing vasculature. To assess the nature of the vascular defects resulting from *Hs6st-2* deficiency, expression of the early vascular development marker *flk-1*, which encodes a VEGF receptor (VEGFR), was examined. Although primary formation of the axial vessels and initial sprouting of intersegmental vessels proceed normally in *Hs6st-2* morphants, the expression of the later vascular development markers *tie-1* and *tie-2* in the caudal vein are reduced during the time period of remodeling. This result correlates with the reduced branching complexity of the caudal vein plexus in the *Hs6st-2* morphants, and expression of *Hs6st-2* at a time point that overlaps angiogenic remodeling, further suggesting that *Hs6st-2* plays an essential role in vascular remodeling of the caudal vein plexus. Therefore, these observations demonstrate that HS is a critical controller of vascular morphogenesis in zebrafish. These observations also illustrate an important role for 6-O-sulfation of HS in vascular morphogenesis.

2. Mouse Studies—Within the last few years, several HS biosynthesis genes, including copolymerase and modification enzymes, have been genetically inactivated in mice, allowing us to determine the general roles as well as the fine structure of HS in the modulation of vascular development physiological conditions close to those in humans. So far, biological functions of HS in vascular development have been examined in mice lacking *Hs6st-1*, *Ndst-1*, *Hepi*, or pericyte-specific *Ext-1* (Table I).

a. *Hsst-1*–/– Mice^{35,36}—Since 6-O-sulfation of HS has been shown to be critical for vascular development in zebrafish, Habuchi et al. further examined the biological functions of *Hs6st* in vascular development in mice, wherein *Hs6st* exists in three isoforms (*Hs6st-1*–*3*) and

one alternatively spliced form (*Hs6st-2s*). Biochemical and expression pattern analyses showed that although the substrate specificities of these isoforms overlap, their expression varies in a tissue and developmental stage-specific manner, suggesting the possibility that the three *Hs6sts* might not have overlapping functions *in vivo* as do those that occur with the *Ndsts*. Among the three *Hs6sts*, *Hs6st-1* carries out the 6-O-sulfation modification of HS in most tissues. The biological and physiological importance of *Hs6st-1* was determined by examining *Hs6st-1* null (*Hs6st-1*^{-/-}) mice.^{35,36} Most of the *Hs6st-1*^{-/-} mice die between embryonic day 15.5 (E15.5) and the perinatal stage, and a low percentage of the mutant mice that survive are considerably smaller than their wild-type littermates (Table I). Some of the *Hs6st-1*^{-/-} mice exhibited developmental abnormalities, including a ~ 50 % reduction in the number of fetal microvessels in the labyrinthine zone of the developing placenta. In support of this, HS structure analysis showed that *Hs6st-1* plays a particularly important role in HS biosynthesis in the placenta. Interestingly, despite the apparent reduction of angiogenesis in the placentas of *Hs6st-1*^{-/-} mice, vascularization in the embryo body, including in the yolk sac, appeared unaffected. These results indicate that the nutritional supply and gas exchange in the labyrinthine zone may be jeopardized in *Hs6st-1*-deficient placentas due to reduced angiogenesis. The reduction in the number of microvessels in the placentas of *Hs6st-1*-deficient embryos suggests the presence of ischemia, which at least partially contributes to the embryonic lethality phenotype of the *Hs6st-1*^{-/-} mice. Taken together, these data demonstrate that 6-O-sulfation of HS is also critically required for vascular morphogenesis in mammals.

b. *Ndst-1*^{-/-} and *Hepi*^{-/-} Mice^{29,32,33} *Ndst* carries out the first step in the modification of HS biosynthesis by replacing the *N*-acetyl group of discrete *N*-acetylglucosamine residues with a sulfate (Fig. 1). This reaction creates the basis for all further modification of the chains in the NS-domains and determines the fine structure of the final HS. Therefore, genetic manipulation of *Ndst* expression represents an effective way to determine the HS structure–function relationship in the regulation of vascular morphogenesis. *Ndst* exists in four isoforms, *Ndst-1*–*4*. Both *Ndst-1* and *Ndst2* are ubiquitously expressed during development and in adult mice, while *Ndst3* and *Ndst4* are mostly expressed during development^{55–57}. Surprisingly, inactivation of *Ndst2* only results in mast deficiency in the connective tissue in the mutant mice (Table I).⁵⁸ In contrast, mice lacking *Ndst-1* (*Ndst-1*^{-/-}) have a severe phenotype and die between E14.5 and shortly after birth (Table I).²⁹ The dramatically reduced HS-sulfation modifications in *Ndst-1*^{-/-} mice results in brain malformation and skeletal defects.^{56,59} In contrast to wild-type hindbrains where vascular endothelium and pericytes are closely associated with tip cells at the outermost front of the growing vascular plexus, a detailed examination of developing vasculature in the E11.5 hindbrain reveals that pericytes in the *Ndst-1*^{-/-} hindbrain frequently failed to reach the growing vascular front and pericytic processes frequently stretched away from the endothelium. This examination shows defective MC spreading and tight attachment during vascular maturation in *Ndst-1*^{-/-} mice.³⁰ Quantification of the proportion of pericyte-endothelial apposition (pericyte coverage) in relation to the total area of the endothelium showed that when compared with wild-type littermates, the pericyte coverage is significantly reduced in the periphery, although not at the midline of the *Ndst-1*^{-/-} hindbrain, thus indicating that the initial recruitment phase is more severely affected by *Ndst-1* deficiency. In summary, these observations established that N-sulfation of HS is critically required for MC recruitment in angiogenesis.

Staining of wild-type hindbrains with the anti-HS antibody HepSS-1 revealed that the most abundant HS deposition is on the abluminal endothelial surface, the interface between ECs and pericytes. In contrast, *Ndst-1*^{-/-} embryos almost completely lacked endothelial HepSS-1 labeling. These results demonstrated that *Ndst-1* is required for endothelial HS-sulfation, and also suggested that this in turn is critical for pericyte recruitment and coverage

in angiogenesis. In endothelium, *Ndst-1* is abundantly expressed. We recently generated EC-specific *Ndst-1* mutant (*EC-Ndst-1*^{-/-}) mice. The targeted inactivation of *Ndst-1* results in endothelial HS with about 50% reduction in N-, 2-, and 6-O-sulfation, showing a globally reduced sulfate modification that is similar to the *Ndst-1*^{-/-} mice.^{31,32} In contrast to the *Ndst-1*^{-/-} mice, the *EC-Ndst-1*^{-/-} mice appear normal and fertile. Detailed examination revealed that vascular morphogenesis is slightly disrupted in some localized areas (Zhang and Wang, unpublished data), consistent with the hypothesis that sulfated domains on endothelial HS regulate vascular morphogenesis *in vivo*. However, it needs to be determined whether endothelial *Ndst-1* deficiency disrupts pericyte recruitment thus contributing to vascular dysmorphogenesis in the *EC-Ndst-1*^{-/-} mice. Since disruption of the *Ndst-1* gene results in only a partial lesion in endothelial HS structure, additional experiments that disrupt endothelial *Ndst-1* and *Ndst2* (or the HS copolymerase *Ext-1* or *Ext-2*) may provide more clear data about the role of endothelial HS in vascular development.

Ndst-1 inactivation reduces multiple sulfation modifications (by ~ 50% over-all).^{31,32} Therefore, the findings upon examination of *Ndst-1*^{-/-} embryos/mice implicate the highly sulfated NS-domains in vascular development, but do not specifically differentiate among requirements for defined sequence or overall charge density. Studies to determine the fine structure requirements for HS in angiogenesis have also merged. As described earlier, 6-O-sulfation of HS is critically required for vascular morphogenesis in mammals.^{35,36} The role of *Hepi* in MC recruitment was examined too.³⁰ In HS biosynthesis, *Hepi* catalyzes the epimerization of GlcA to IdoA, which increases the flexibility of the sugar chain, and is also shown to be a prerequisite to some of the further steps in O-sulfation. Deletion of the *Hepi* gene leads to the formation of a mutant HS that is highly N-sulfated, but devoid of IdoA units, and is characterized by a severely perturbed fine structure.³³ Analysis of MC recruitment and coverage in *Hepi*^{-/-} embryos revealed only a transient delay in MC recruitment in the hindbrains of E10.5 *Hepi*^{-/-} embryos with no sustained defects. Therefore, this study illustrated that unlike N- and 6-O-sulfation, C5-epimerization appears not to be critical for MC recruitment in vascular development.

c. Mural Cell-Specific *Ext-1* Null (*MC-Ext-1*^{-/-}) Mice²⁶: The *Ext-1* gene encodes for the key glycosyltransferase that is essential for elongation of the HS disaccharide backbone chain (Fig. 1). Deletion of *Ext-1* leads to a complete loss of HS, and therefore can determine the general role of HS in physiological/ pathological processes.⁶⁰⁻⁶² To study the functional role of MC-derived HS in angiogenesis, *Ext-1* was selectively inactivated in mouse MCs (*MC-Ext-1*^{-/-}).²⁶ Anti-HS antibody staining confirmed substantial loss of HS in the MC compartment of major arteries and veins, and in smaller vessels as well as MC progenitors in *MC-Ext-1*^{-/-} embryos, allowing one to address the functional importance of MC-specific HS in vascular development. *MC-Ext-1*^{-/-} embryos appear to develop normally by E11.5, but die between E13.5 and E19 with hemorrhages and edema in the skin due to increase vessel permeability.²⁶ Detailed wholemount staining for ECs of the skin reveals striking abnormalities in superficial vessels, particularly around the spinal cord in the *MC-Ext-1*^{-/-} embryos. In contrast to the wild-type controls that display an organized and hierarchical vascular pattern, vessels in *MC-Ext-1*^{-/-} embryos appear random and chaotically branched with variable diameters, and frequently form glomeruloid structures resembling microaneurysms. Detailed examination further observed significant increases in vessel regression, diameter variability, and permeability in the *MC-Ext-1*^{-/-} skin tissues.²⁶ Intriguingly, the central cardiovascular development and function appear normal in *MC-Ext-1*^{-/-} embryos, indicating that the observed vascular defects in the skin reflect local defects associated with MC HS deficiency.

Examination of MCs in skin samples revealed that in the control embryos, MCs are closely attached to the endothelium and cover almost the entire vascular network. The *MC-Ext-1*^{-/-}

skin samples from identical regions showed significantly reduced MC coverage, with many vessels lacking properly attached MCs.²⁶ In parallel, examination of isolated NG2-positive cells, which represent MC progenitor cells not yet recruited to the vessels or MCs that have detached, showed that the isolated NG2 positive cells are more abundant in *MC-Ext-1*^{-/-} skin than in the wild-type control.²⁶ Morphologically, the isolated NG2-positive MCs appear spindle-shaped in wild type, while they are conspicuously round in the mutant, revealing a MC–EC attachment defect. Taken together, these observations demonstrate that MC HS is critically required for MC cell recruitment as well as for maturation and stabilization of nascent vessels during development.

D. Heparan Sulfate in Developmental Angiogenesis: Modulation of Growth Factor Signaling

Although HS has been shown to facilitate the biological functions of both pro- and antiangiogenic factors, the study of HS mutants using both zebrafish and mouse models illustrates that HS plays a proangiogenic role during developmental angiogenesis. Therefore, the predominant effect of endothelial HS in this setting is to facilitate the actions of proangiogenic factors that promote developmental angiogenesis. So far, studies of *Hs6st-2*^{-/-} – zebrafish and mice lacking *Hs6st-1*, *Ndst-1*, *Hepi*, or *MC-Ext-1* reveal that HS critically modulates VEGF-A, PDGF-B, and TGF- β signaling to promote the various steps in developmental angiogenesis.

1. VEGF-A Signaling—Genetic studies established that VEGF-A is a master regulator of developmental angiogenesis as a result of its ability to modulate almost all aspects of the process, including EC differentiation, assembly, proliferation, and migration (Table I).^{1,2,8,63} VEGF-A is expressed and secreted as multiple, homodimeric isoforms that are formed as a result of differential splicing of the VEGF pre-mRNA. The most abundantly expressed isoforms are VEGF120, VEGF164, VEGF188 in mice, and VEGF121, VEGF165, and VEGF189 in humans. Although all variants contain the same binding sites for the VEGFR, they differ in their affinity for HS because of the presence or absence of HS-binding domains that are encoded by exons 6 and 7.^{40,41} *In vivo*, both domains independently mediate interactions with HS present on the cell surface and in the ECM. VEGF120, which lacks the two HS-binding domains, is freely diffusible in ECM, whereas VEGF164 and VEGF188 exhibit moderate and high binding affinity, respectively, resulting in partial or complete retention of these isoforms in HS-rich compartments.^{39–41} Meanwhile, studies also observed that these isoforms differ in their mitogenicity, chemotactic properties, receptor-binding characteristics, and tissue-specific expression,^{64–67} suggesting that HS may differentially modulate the biological functions of VEGF isoforms *in vivo*. This was tested by examination of mice expressing exclusively the VEGF120 isoform (VEGF120/120 mice).³⁹ The most dramatic phenotype of the VEGF120/120 mice is ischemic cardiomyopathy, resulting from the impaired postnatal myocardial angiogenesis. The VEGF120/120 mice ultimately die of cardiac failure.³⁹ Detailed examination of vascular development in VEGF120/120 embryos further revealed that, although heparin-binding VEGF-A is not essential for vasculogenesis or angiogenesis, the mutant shows reduced vascular branching complexity with increased microvessel caliber,^{39–41} suggesting that the ability to bind HS is necessary for VEGF-A to regulate vascular branching. This observation also suggests that HS interacts with VEGF-A to modulate vascular development *in vivo*.

The regulatory role of HS in VEGF-A signaling *in vivo* was examined in zebrafish by injection or coinjection of morpholinos against *Hs6st-2* and VEGF-A.³⁴ Compared with a single injection of either *Hs6st-2* MO or VEGF-A MO alone, coinjection of *Hs6st-2* MO and VEGF-A MO resulted in a synergistic increase in the frequency of embryos with caudal vein branching defects. Meanwhile, coinjection of *Hs6st-2* and a four-base mismatch VEGF-A

MO abolished the synergy. These results illustrate that *Hs6st-2* and *VEGF-A* interact *in vivo* during caudal vein formation, also establishing that HS facilitates VEGF-A signaling in angiogenesis *in vivo*.

Studies have also addressed the mechanism of how HS modulates VEGF-A signaling. The detailed analysis of vascular branching patterns, EC behavior, and VEGF-A protein distribution in wild-type mouse embryos and mouse mutants producing solely VEGF120 or VEGF188 revealed that the heparin-binding VEGF-A isoforms serve as spatially restricted stimulatory cues that elicit stereotypical branching behavior at the leading edge of the growing microvessel network,^{39–41} an analogy to the role of HS localizing FGF and BMP in other branching organs. These observations strongly suggest that HS functions to maintain a VEGF-A concentration gradient that provides spatially restricted stimulatory cues to polarize and thereby guide sprouting ECs to initiate vascular branch formation. Meanwhile, *in vitro* EC culture studies established that HS functions *in cis* as a cell-surface coreceptor for VEGF-A signaling. Recent *in vitro* studies using chimeric cultures of embryonic stem cells defective in either HS production (combined deficiency of *Ndst-1* and *Ndst-2* resulting in cells deficient in *N*-sulfate, 2-O-sulfate, and 6-O-sulfate) or VEGFR2 synthesis demonstrated that VEGF signaling in ECs is fully supported by HS expressed *in trans* by adjacent MCs, suggesting a mechanism whereby HS *in trans* potentiates VEGF-A signaling during developmental angiogenesis.⁶⁸ However, VEGF-A signaling in ECs in *MC-Ext-1*^{-/-} embryos is not altered,²⁶ indicating that MC HS is not required for VEGF-A signaling during vessel development *in vivo*. This observation also highlights the importance of determining the requirement of endothelial HS for VEGF-A signaling in vascular development *in vivo*.

2. PDGF-B Signaling—Deletion of PDGF-B and PDGF receptor (PDGFR- β) causes embryonic lethality resulting from vascular defects, including microaneurysms, vascular leakage, and hemorrhaging as a result of severe MC loss, establishing that PDGF signaling is pivotal for MC recruitment, vascular maturation, and stability.^{44–46} Conditional endothelial inactivation of PDGF-B established that the endothelium provides the major source of PDGF-B required for the migration and proliferation of MCs.⁴⁶ PDGF-B protein is secreted as PDGF-BB homodimers. PDGF-BB binding to PDGFR- β on MCs leads to receptor dimerization and phosphorylation of PDGFR- β , activating multiple downstream signaling pathways, including PI3K and Erk, which then stimulate cell migration and proliferation. PDGF-B contains a conserved C-terminal sequence of basic amino acids (retention motif), which is structurally similar to the HS-interacting domain of VEGF-A and is thought to mediate PDGF-B binding to the cell surface or ECM.^{69,70} Mice deficient in the PDGF-B retention motif (*PDGF-B^{ret/ret}*) display vascular defects associated with MC detachment (Table I),⁴⁴ which is reminiscent of the MC recruitment defects observed in *Ndst-1*^{-/-} and *MC-Ext-1*^{-/-} mice, suggesting that HS facilitates PDGF-B signaling to recruit MCs during developmental angiogenesis. Protein lysates from *MC-Ext-1*^{-/-} skin samples showed a strong reduction in phosphorylated PDGFR- β , and loss of SHP2 and Erk1/2 activation. These observations reveal that MC HS participates in MC recruitment by facilitating PDGFR- β signaling in skin. Interestingly, MCs in the CNS do not require HS production for effective PDGFR- β signaling and recruitment to the vessel wall. The differential requirement of HS may result from different modes of MC recruitment in different tissues. In CNS, the MC recruitment occurs via a longitudinal model, which relies on migration and proliferation of MCs along the abluminal endothelial surface, and may critically require endothelial HS to control PDGF-B retention and to function as a coreceptor for pericytic PDGFR- β activation *in trans*,⁷¹ whereas the skin recruitment of MC to vessels involves induction of new progenitor cells from mesenchymal lineages. MC progenitor cells that reside at a distance from the vessel may cell-autonomously require HS on the cell surface for activation *in cis*.⁷¹ Therefore, the spatial relationship between the signal-sending

cell (the endothelium) and the signal-receiving cell (the MC) in skin is different from CNS during MC recruitment, explaining the difference in vascular phenotypes of skin and CNS in *MC-Ext-1*^{-/-} mice.²⁶ Meanwhile, this prospective has been supported by studies on developmental and tumor angiogenesis showing that HS retains PDGF-B close to the EC surface to facilitate directed MC migration along the sprouting vessel and to mediate proper MC attachment to the vessel during angiogenesis.^{44,46} Therefore, it would be interesting to see whether endothelial HS deficiency disrupts PDGF-B signaling and the PDGF-B-mediated MC recruitment, contributing to the vascular development defects in *EC-Ndst*^{-/-} mice that we maintain in the laboratory.

The fine structure of HS required for PDGFR- β signaling *in vivo* was examined in *Ndst-1*^{-/-} and *Hepi*^{-/-} mice.³⁰ Reduction of *N*-sulfation due to deficiency in *Ndst-1* attenuated PDGF-B binding *in vitro* and led to pericyte detachment and delayed pericyte migration *in vivo*. Reduced *N*-sulfation also impaired PDGF-B signaling and directed cell migration, but not proliferation. In contrast, HS from *Hepi*^{-/-} mutants, which is extensively *N*-sulfated, but lacks 2-*O* and 6-*O* sulfation, retained PDGF-B *in vitro* and showed that pericyte recruitment *in vivo* was only transiently delayed. On the basis of these *in vitro* as well as *in vivo* studies, it appears that pericyte recruitment requires HS with sufficiently extended and appropriately spaced *N*-sulfated domains to retain PDGF-B and to activate PDGFR- β signaling, whereas the specific sequence of monosaccharide and sulfate residues does not appear to be important for this interaction.³⁰ Currently, it is believed that HS functions to retain a PDGF-B gradient to direct MC progenitor cell migration and as a coreceptor for PDGFR- β signaling,²⁶ as seen for VEGF-A and FGF.

3. TGF- β Signaling—*Ex vivo* studies using ECs and 10T1/2 cell (MC progenitor cells) coculture illustrated that TGF- β is also critically involved in MC recruitment and differentiation.^{72,73} This *ex vivo* observation was confirmed by genetic studies showing that deficiency in the TGF- β receptor II results in mouse embryonic lethality with defects in vascular development. Meanwhile, mice bearing mutations in the TGF- β signaling pathway, such as TGFRII, Alk1, Alk5, endoglin, and SMAD1/5 mutants, exhibit defective vascular development associated with reduced MC recruitment and impaired vessel stability, demonstrating that TGF- β signaling is essentially required for vascular development. Detailed studies further revealed that TGF- β critically regulates EC and MC proliferation and differentiation, and mediates the *de novo* induction of MCs from the mesenchymal lineage state during embryonic development. TGF- β also induces actin reorganization by activating Rho-GTPases through phosphorylation of SMAD 2 and 3,⁷⁴ which may modulate MC attachment to ECs as well as EC responses. The components of the TGF- β pathway, including TGF- β receptors, interact and cocluster directly with VE-cadherin at EC-EC junctions, suggesting that TGF- β signaling may promote vessel stabilization and quiescence.⁷⁵ TGF- β is known to bind HS,^{76,77} and HS plays an important role in linking the latent TGF- β -binding protein LTBP1 to fibronectin, providing a mechanism for TGF- β storage in the ECM. During the patterning process in *Drosophila*, HS has been proven to be essential for gradient formation and extracellular transport of the TGF- β family member Dpp, suggesting that HS may be important for the availability, potential gradient formation, and activity of TGF- β during the induction and recruitment of MCs. In *MC-Ext-1*^{-/-} embryonic skin vasculature, TGF- β signaling is defective in ECs.²⁶ TGF- β is expressed by both ECs and MCs, and reciprocal signaling regulates MC induction from mesenchymal progenitors as well as various EC functions, including expression of stabilizing matrix components, cell proliferation, and differentiation. Therefore, the disruption of the TGF- β signaling in ECs may contribute to the vascular development defect in the *MC-Ext-1*^{-/-} embryo.

4. Additional Signaling Pathways—In addition to VEGF, PDGF, and TGF- β signaling, other angiogenic pathways may also be modulated by HS during development. This may include FGF, ephrin, slit/roundabout, the netrin/UNC (uncoordinated) receptor, Wnt, hedgehog, and sprouty, as well as ECM components such as fibronectin and laminin. For example, while the *Hs6st-1*^{-/-} mouse placenta had a normal level of *Wnt2* expression, HS from the same placenta showed attenuated binding affinity to Wnt2.³⁵ In agreement, *Hs6st-1* in zebrafish appears to control Wnt-dependent signaling pathways.³⁴ Wnt signaling has been known to induce VEGF-A expression, a strong inducer of angiogenesis.⁷⁹ Attenuated Wnt2-HS interaction in *Hs6st-1*^{-/-} mice may cause decreased VEGF-A expression that is observed in the mutant placentas.³⁵ More work is needed to determine whether the interactions of HS with these growth factors, morphogens, and ECM components are biologically significant in angiogenesis *in vivo*.

E. Heparan Sulfate in Adult Physiological Angiogenesis

1. Reproductive Angiogenesis—The female reproductive organs (ovary, uterus, and placenta) are some of the few adult tissues that exhibit regular intervals of rapid growth. They are highly vascularized, and angiogenesis is an important component of the growth and function of these tissues. As with many other tissues, VEGFs and FGFs appear to be the major angiogenic factors in the female reproductive organs with apparently abundant coexpression of HS.^{35,38} Therefore, it is highly possible that HS may critically modulate reproductive angiogenesis by facilitating VEGF and/or FGF signaling. Although so far no studies have specifically addressed this issue, the phenotypes displayed by *Hs3st-1*^{-/-} and *Hs6st-1*^{-/-} mice appear to strongly support this speculation.^{35,37,38}

Hs3sts carry out the last step of the sulfate modifications that occur during HS biosynthesis (Fig. 1). This occurs through the addition of 3-O-sulfate groups to N-sulfated glucosamine residues. Six *Hs3st* isoforms have been identified with different tissue expression patterns and acceptor substrate specificities. The *Hs3st-1* is the predominant form generating antithrombin-binding sites in anticoagulant HS, particularly in ECs.³⁷ Unexpectedly, *Hs3st-1*^{-/-} mice did not show a procoagulant phenotype, but instead suffered severely compromised fertility.^{37,38} Phenotypic characterization revealed that the female *Hs3st-1*^{-/-} mice exhibited defective reproductive performance in different degrees, including impaired ovarian function as well as intrauterine growth restriction that were linked to delayed placental development.^{37,38} Histological analysis of implantation sites at mid-gestation (E9.5–11.5) further revealed that placental development in the *Hs3st-1*^{-/-} maternal uterus was markedly delayed, with typically increased numbers of trophoblast giant cells, a rudimentary labyrinth, and no patent maternal–fetal circulation (Table I). The lack of blood circulation highlights that angiogenesis is disrupted at the implantation sites of the *Hs3st-1*^{-/-} female uterus, which may contribute significantly to the compromised fertility of *Hs3st-1*^{-/-} female mice. However, further studies are required to determine whether the *Hs3st-1* deletion disrupts angiogenic signaling in the mutant mice. The study of *Hs6st-1*^{-/-} mice has provided further supportive evidence as well.³⁵ The *Hs6st-1*^{-/-} mice exhibit ~50% reduction in the number of fetal microvessels in the labyrinthine zone of the placenta relative to that in wild-type littermates. The reduced vascularization leads to hypoxia, signifying that altered angiogenesis may impair the function of the *Hs6st-1*^{-/-} placentas, which, as a consequence, may contribute to growth retardation and lethality in the *Hs6st-1*^{-/-} embryos. Moreover, the expression of VEGF-A mRNA and its protein is reduced in the *Hs6st-1*^{-/-} placenta, highlighting that HS regulates VEGF-A signaling to modulate vascular development in the placenta.³⁵ However, our *EC-Ndst-1*^{-/-} mutants develop normally with normal fertility,^{31,32} indicating that a more severe HS structure alteration in the vasculature is required to rigorously test whether HS modulates reproductive angiogenesis.

2. Wound Angiogenesis—Wound healing is a complex tissue remodeling process, and may be conveniently divided into three phases: inflammatory, proliferative, and remodeling. Leukocytes that infiltrate tissues during the inflammatory phase secrete proangiogenic growth factors and cytokines that recruit ECs to initiate angiogenesis, a major feature of the proliferative phase. Angiogenesis is essential for wound healing. Newly formed blood vessels participate in provisional granulation-tissue formation, and provide nutrition and oxygen to the growing tissues. Wound angiogenesis is tightly regulated by diverse HS-binding factors, including VEGF, FGF, TGF- β , PDGF-B, SDF-1, and MCP-1, which act in sequential, concerted, and synergistic manners, suggesting that HS may modulate the functions of these protein ligands to critically regulate wound angiogenesis. This hypothesis has been strongly supported by studies of *syndecan-4*^{-/-} and heparinase-overexpressing transgenic mice.^{80,81}

The syndecans are a family of transmembrane HS-proteoglycans. Syndecans have been shown to bind proangiogenic factors and cytokines to regulate the angiogenic process.¹⁴ Syndecan-4 is detectable in the epidermis, but not in the dermis, of uninjured adult mouse skin. After skin injury, however, syndecan-4 is upregulated throughout the granulation tissue on fibroblasts and ECs, suggesting that syndecan-4 may regulate wound healing and related angiogenesis. This was tested by the examination of *syndecan-4* mutant mice. The *syndecan-4*^{-/-} and *syndecan-4*^{+/-} mice were viable, fertile, and macroscopically indistinguishable from wild-type littermates, but showed statistically significant delayed healing of skin wounds and impaired angiogenesis in the granulation tissue, thus demonstrating directly that syndecan-4 is an important cell-surface receptor in wound healing and wound angiogenesis. Most of the biological functions of syndecans are mediated by their HS moiety,¹⁴ suggesting that HS critically regulates wound angiogenesis. This is alternatively supported by examining wound healing in heparinase-overexpressing mice.⁸¹ Heparanase, an endoglycosidase that degrades HS in the ECM and at cell surface, releases HS-bound growth factors and converts them into bioactive molecules. Heparanase is expressed in wound granulation tissue, suggesting that heparanase may modulate the bioavailability and activation of a multitude of mediators capable of promoting EC migration, proliferation, and MC recruitment in the complex setting of wound healing. Indeed, heparanase-overexpressing transgenic mice showed a remarkably elevated wound angiogenesis response.⁸¹ Consistent with this, topical application of recombinant heparanase significantly accelerated wound healing and markedly improved wound survival, which then proceeded with enhanced blood vessel maturation.⁸¹ These observations clearly demonstrated that heparanase-accelerated tissue repair and skin survival occur through enhancement of the angiogenic response, further supporting the importance of HS in the modulation of wound angiogenesis. In a recent study, where tumor angiogenesis was altered in the EC- *Ndst-1*^{-/-} state, we noted that wound angiogenesis was not obviously altered in comparison with that of wild-type littermates,³¹ showing that endothelial *Ndst-1* may be dispensable for wound healing and wound angiogenesis. Given that endothelial *Ndst-1* ablation only partially alters HS structure, more dramatic lesions in HS structure may be required to determine the requirement and the role of HS in wound angiogenesis.

II. Endothelial Heparan Sulfate in Pathologic Angiogenesis

A. Tumor, Vasculature, Stroma, and Heparan Sulfate: An Introduction

A large body of work has now highlighted a number of important roles that HS plays in the formation, vascularization, and spread of solid tumors. It is important to recognize at the outset that an important biological principle is that solid tumors and their metastases must gain a vasculature if they are to grow into macroscopic tumors. The threshold for nonvascular growth is typically ~ 1 mm. Beyond that size, tumors must be remodeled through the process of angiogenesis in order to grow (and contribute to multiple downstream

pathologic consequences). The focus herein is on the roles of vascular endothelial HS in the growth and remodeling of tumor neovasculature. A practical way to discuss HSPGs produced by both nascent as well as established tumor vasculature is by categorizing them into either EC-surface HSPGs that remain tethered as part of the endothelial glycocalyx or secreted HSPGs distributed in the perivascular ECM, including those that contribute to the content of vascular basement membranes. It should be noted that production of the latter might result from HSPGs secreted by not solely the vascular endothelium, but by tumor and/or stromal cells, including perivascular cells, in the same microenvironment. We shall consider these anatomical relationships as we discuss the forces that regulate tumor vascular HSPGs and their interactions with multiple tumor vascular effector molecules and consider the effects of targeting genetic alterations in HSPG biosynthesis to distinct cellular compartments in models of tumor angiogenesis.

B. Endothelial Heparan Sulfate and the Control of Tumor Proangiogenic Growth Factors

1. Tumor Hypoxia, Heparan Sulfate, and the Distribution of Tumor

Proangiogenic Growth Factors—During states of hypoxia, expression of the hypoxia-inducible transcription factor (HIF-1) stimulates the expression of VEGF, which leads to responses that promote oxygen delivery to hypoxic tissues, such as increases in vascular permeability and capillary density as a result of angiogenesis.^{83,84} In neoplasia, this process is stimulated by rapid clonal tumor growth, with associated hypoxia as the tumor mass rapidly outgrows its blood supply. In addition, data from mice bearing an endothelial-specific genetic alteration in HIF-1 α shows that HIF-1 expression is also important in sustaining an autocrine signaling loop involving VEGF and the major endothelial receptor VEGFR-2.⁸⁵ Through its ability to bind distinct isoforms of VEGF, endothelial HS may play important roles in regulating matrix as well as EC-surface distribution of VEGF during this process. The biosynthesis of VEGF, also known as VEGF-A to distinguish it from other VEGF homologs involved in cardiac and lymphatic development, is posttranscriptionally regulated. A variety of splice variants, including VEGF145, VEGF165, and VEGF189, retain basic-amino acid rich C-terminal exons (6 and/or 7) that confer heparin-binding ability.⁸⁶ HS expressed on EC-surface proteoglycans of growing tumor microvasculature as well as proteoglycans secreted into the ECM has the ability to differentially bind, and thereby control gradients, of these potent proangiogenic VEGF isoforms.⁸⁷ This is in addition to its ability to control gradients of several other growth factors (to be discussed further), allowing the distribution and/or mobilization of several important proangiogenic factors to be modulated by the production and distribution of HS. In the case of VEGF-A, the distribution of heparin-binding species of VEGF (e.g., VEGF165 or VEGF189) is distinct from that of non-HS binding species such as VEGF121, wherein the latter may diffuse to a further distance from tumor cells, thereby affecting vasculature at a greater distance from VEGF-secreting tumor nests.⁸⁷ Moreover, the development of hypoxia within the center of rapidly growing tumors may affect both the biosynthesis of HS and the way in which HS interacts with these factors, thereby adding an additional layer growth factor modulation. For example, hypoxia increased FGF2-binding sites on HS as well as the action of FGF-2 over that observed in normoxic condx.⁸⁸ Moreover, with respect to VEGF, it is interesting that cancer microenvironment changes in pH appear to affect the expression of VEGF splice variants, with greater expression of VEGF121 by tumor cells exposed to a more acidic environment.⁸⁹ This appears to be associated with p38 signal activation and SR protein (splicing factor) expression and phosphorylation. Since hypoxia results in anaerobic metabolism and tissue acidemia, it is possible that differential release of distinct VEGF isoforms varies with distance from the hypoxic tumor center. Thus, the hypoxic tumor center may produce a more “diffusible” form of VEGF-A (121 isoform), leaving the tumor center with a relative paucity of (matrix-bound) VEGF. Whether this is a major factor contributing to a progressive lack of blood supply and necrosis of the tumor center remains

to be determined. Nevertheless, a common theme in the pathophysiology of tumor angiogenesis is the establishment of proangiogenic growth factor gradients that appear to be induced and controlled by both hypoxia as well as the expression of HS, with the latter possibly having profound effects on the distribution of major growth factors and the consequent patterns of tumor vascularization.

2. Multiple Heparin-Binding Growth Factors and the “Redundancy” of Proangiogenic Effectors—

The targeting of VEGF-A in human tumors using a humanized anti-VEGF antibody has become standard practice for advanced-stage tumors of the lung and colon.^{90,91} The effects of this on tumor vasculature include regression as well as narrowing and smooth-muscle investment of the tumor’s tortuous vasculature network, with the latter resulting from the effects of high tumor VEGF-A levels on tumor-associated endothelium.⁸ In lung cancer, adding this form of anti-VEGF therapy to combination chemotherapy results in a modest survival advantage⁹¹; however, a variety of mechanisms may limit efficacy and duration of the effect, including induction of other proangiogenic factors such as PDGF and FGF-2 (reviewed in Ref. 92). Targeting HS may alter receptor binding by several heparin-binding proangiogenic growth factors that may be induced in tumors (including HGF, PDGF, FGF-2, among others). Thus, it is possible that the escape from VEGF-blockade as a result of such redundant tumor growth mechanisms may be subverted by targeting endothelial HS biosynthesis. One method to achieve this may be through the introduction of polysulfated heparin mimetics or polysulfonated compounds (e.g., suramin) that may compete for binding to FGF-2⁹³ and possibly multiple growth factor–HS interactions during tumor angiogenesis. Yet another potential form of anti-VEGF resistance is the persistence of basement membrane “ghosts” following VEGF blockade.^{94,95} It has been demonstrated that tumor endothelium may grow back to fill these channels once VEGF-therapy is stopped or held.⁹⁵ Basement membranes are endowed with abundant secreted HS-proteoglycans that may bind and store high levels of FGF-2, VEGF-A, and other proangiogenic factors. It is thus possible that targeting HS in this setting may prevent such regrowth following anti-VEGF therapy.

3. Regulation of Endothelial Heparan Sulfate in Cancer: Heparanase and Sulfatases

a. Heparanase: Storage of growth factors bound to HS in tumor ECM as well as vascular basement membranes provides a “bank” of proangiogenic factors that may be mobilized by the release of heparanase by tumor cells.⁹⁶ Mammalian heparanase is an endo-beta-d glucuronidase that degrades HS ubiquitously expressed on cell-surface as well as matrix-associated proteoglycans. Homozygous transgenic mice that overexpress heparanase demonstrate both a profound reduction in the size of HS chains as well as excess branching of mammary gland ducts and enhanced neovascularization of mammary ducts as well as hair follicles. The overexpression of heparanase by tumors may activate tumor angiogenesis through a variety of mechanisms in addition to promoting the release of growth factor-decorated HS fragments.^{96,97} It now appears that transgenic (including tumor-induced) expression of heparanase leads to increased HS *N*- and *O*-sulfation, which in turn strongly promotes the formation of FGF-1 or FGF-2—FGFR-1 ternary complexes.⁹⁸ This may also promote HS coreceptor activity for other vascular endothelial growth factors. In addition, the action of heparanase in myeloma as well as other tumors promotes the shedding of syndecan-1, which in turn complexes with VEGF-A in the ECM that in turn facilitate growth and migration of ECs through the combined ability of VEGF-A/syndecan-1 complexes to activate endothelial VEGFRs and engage integrins through an integrin-binding region on the syndecan-1 core protein.^{99,100} Accordingly, inhibition of heparinase has become appealing as a form of antiangiogenic therapy. Along those lines, a variety of nonanticoagulant forms of heparin have been developed as competitive inhibitors of

mammalian heparinase.¹⁰¹ Novel inhibitors of heparanase that may inhibit tumor angiogenesis include polysulfated penta and tetrasaccharide glycosides¹⁰² as well as *N*-acetylated glycol-split heparin species.¹⁰³

b. Sulfatases: A growing literature has revealed the importance of another family of enzymes that modifies the structure of mature HS chains in carcinoma: the extracellular sulfatases Sulf-1 and Sulf-2. As endosulfatases, these enzymes remove internal glucosamine 6-*O*-sulfate modifications within HS.¹⁰⁴ A variety of tumors, including ovarian, breast, and hepatocellular carcinomas, appear to downregulate Sulf-1 expression, which in turn appears to upregulate autocrine and/or paracrine tumor-cell signaling by a variety of HS-binding tumor growth factors, including FGF-2, HGF, and HB-EGF.^{105,106} The importance of these enzymes in tumor angiogenesis has been examined in a few preliminary studies. Curiously, while short hairpin RNA-mediated inhibition of Sulf-1 in human ECs was associated with increased FGF-2, HGF, and VEGF165-mediated endothelial proliferation,¹⁰⁶ another study demonstrated that the exogenous addition of purified recombinant Sulf-2 promoted angiogenesis in the chick chorioallantoic membrane assay.¹⁰⁷ One explanation for these apparently contradictory findings may center on the spatial context or site of action of the enzyme (i.e., removal of glucosamine 6-*O*-sulfate on ECM HS vs. cell-surface HS). Specifically, the effect may vary with respect to how the enzyme is manipulated during the experiment. In this manner, it is possible that inhibiting or silencing endothelial production of the enzyme may exert its dominant effect on cell-surface HS, thereby promoting HS glucosamine 6-*O*-sulfate-dependent receptor activation by FGF-2,^{101,106} whereas exogenous addition of enzyme to the angiogenesis system may have preferential effects on reducing glucosamine 6-*O*-sulfation on ECM HS, thereby increasing the availability of matrix-bound growth factor for binding to endothelial growth receptors.¹⁰⁷ Further studies are needed to define the specific effects of the sulfatases on tumor endothelium and understand the interplay of those effects with the well-characterized roles of sulfatase expression on tumor-cell growth and behavior in the same microenvironment.

C. Targeting HSPG Mutations to Distinct Vascular Cells in Cancer Models: Lessons Learned

1. Models Targeting Endothelial Heparan Sulfate Biosynthesis—Data acquired from both developmental as well as tumor-based studies in mice employing endothelial-targeted genetic alterations in HS biosynthesis have improved our understanding of the importance of HS in tumor angiogenesis. One approach is to attempt the targeting of HS polymerization in the mouse, with the aim to block HS chain assembly on all HS-proteoglycans either systemically or targeted to the endothelium. The goal would then be to examine tumor growth and vascularization on the mutant background. This has been limited by either arrest of development during gastrulation (including lack of mesoderm development) in embryos harboring a homozygous-null mutation in the HS copolymerase *Ext-1*,⁶² or embryonic lethality encountered upon attempting to drive the *Ext-1* mutation specifically in ECs by crossing conditional (floxed) *Ext-1* mutant mice onto the *Tie2Cre* transgenic background (unpublished data). Using chimeric cultures of embryonic stem cells bearing alterations in either the HS or VEGFR-2 biosynthetic pathways, Jakobsson and others⁶⁸ were able to demonstrate that endothelial signaling in proliferating vasculature may be fully supported by HS acting *in trans* by adjacent supporting smooth-muscle perivascular cells. The degree to which this mechanism might contribute to endothelial growth and remodeling during tumor angiogenesis is unknown; however, targeting biosynthesis of the major HS sulfating enzyme *N*-deacetylase/*N*-sulfotransferase-1 (*Ndst-1*) to ECs (i.e., *Ndst-1* *ff* *Tie2Cre* mutants) is sufficient to alter tumor growth and angiogenesis in adult mutants.³¹ Mutant tumor vasculature demonstrated abnormalities in branching as well as alterations in its ability to bind and signal in response to FGF-2 and VEGF165. Interestingly, our group

has since discovered that marked inhibition of tumor growth angiogenesis is achieved in mice bearing the same endothelial-targeted mutation on a transgenic spontaneous mammary carcinoma background (M. M. Fuster, unpublished data). It thus appears that the cell-autonomous action of appropriately sulfated tumor endothelial HS is required to facilitate the action of major proangiogenic tumor growth factors in tumor angiogenesis. More work is needed to better understand the additional importance of HS produced by other cells, including tumor, mural, and other stromal cells, during tumor angiogenesis.

2. Models Targeting Pericyte Heparan Sulfate Biosynthesis—A number of studies now highlight important functions served by HS produced by vascular pericytes during endothelial sprouting and remodeling. However, most of these studies have focused on vascular growth and remodeling during development and not tumor angiogenesis *per se*. Nevertheless, understanding the genetic importance of “periendothelial” HS in this context may provide valuable clues for future studies examining the genetic importance of pericyte HS in tumor angiogenesis. A key heparin-binding growth factor that has been implicated in the recruitment of pericytes onto angiogenic vasculature is endothelial PDGF-BB. Deleting the C-terminal HS-binding motif of PDGF-BB results in impaired vascular retention of the growth factor and impaired pericyte recruitment. Studies examining pericyte recruitment in gene-targeted mice bearing alterations in HS biosynthesis, including *Ndst-1* mutants, showed that recruitment of pericytes requires glucosamine *N*-sulfated domains to activate the receptor PDGF-R-beta by retained PDGF-BB.³⁰ In carcinomas, the effect of altering PDGF binding and function through the targeting of HS may be complex. First, in tumor models, PDGF-BB appears to stimulate pericyte recruitment through induction of the endothelial chemokine stromal-derived factor-1 alpha (SDF1alpha), and CXCR4-mediated pericyte chemotaxis.¹⁰⁸ Although this may stimulate the release of proangiogenic mediators released from pericytes,¹⁰⁹ it also supports vessel maturation by investing newly formed endothelial tubes with VSMC pericytes. Interestingly, the latter phenomenon in tumors appears to occur during “normalization” or “maturation” of tumor vessels as a result of anti-VEGF therapy in cancer,⁸ wherein pericyte investment occurs during a “straightening” and narrowing of the tortuous tumor vasculature during VEGF blockade. This in turn appears to result in reduced tumor interstitial pressure and more favorable hemodynamic conditions for the delivery chemotherapy. In addition, a recent study demonstrated that VEGF was found to negatively regulate the effects of PDGF on pericytes through the formation of novel receptor complexes made up of activated VEGFR-2 and PDGF-R-beta.¹¹⁰ In a fibrosarcoma model, VEGF(–/–) sarcomas were associated with a reduction in VEGFR-2/PDGF-R-beta complexes when compared with that in VEGF(+ / +) sarcomas, and this was in turn associated with tumor vessel maturation.¹¹⁰ Thus, although endothelial HS alterations may attenuate angiogenic growth signaling in ECs, the effects of altering HS on pericytes during tumor angiogenesis may be more complex, wherein an inhibitory effect on the (VEGF-mediated) release of proangiogenic growth factors by pericytes may be balanced by an alteration in PDGF-mediated pericyte recruitment, and the associated maintenance of a tortuous, immature vessel that lacks smooth-muscle investment. Further genetic studies targeting pericyte HS during tumor angiogenesis may reveal the “balance” of these factors on tumor vascular growth and hemodynamics.

3. Models Targeting the Scaffolding of Heparan Sulfate Core Proteins—

Studying the effects of altering HS biosynthesis in endothelium has led to important insights with respect to the coreceptor- and growth factor-sequestering functions of HS in tumor angiogenesis, and several interesting patterns have emerged from genetic studies targeting HS proteoglycan core proteins. As protein scaffolds for HS chains on EC membranes, members of the syndecan and glypican core protein families (reviewed in Ref.111) may serve in a cell-autonomous manner to facilitate receptor binding and signaling by major

proangiogenic growth factors to growing tumor endothelium. Athymic mice lacking glypican-1 demonstrate decreased tumor angiogenesis in response to intrapancreatic implantation of PANC-1 or T3M4 human pancreatic cancer cells. Syndecan-4 knockout mice demonstrate defects in wound angiogenesis,¹¹² although the specific importance of syndecan-4 in tumor angiogenesis has not been reported. The effects of syndecan-4 deletion on endothelial behavior may not solely be due to the associated reduction in EC-surface HS (i.e., as a coreceptor), but may also have to do with the finding that syndecan-4 plays an important role in focal adhesion kinase phosphorylation and cell migration. In tumor models, genetic studies have also recently revealed a domain within the endothelial syndecan-1 core protein that directly associates with $\alpha(v)\beta(3)$ and $\alpha(v)\beta(5)$ integrins, and facilitates integrin-mediated tumor angiogenesis.¹¹⁴ Finally, secreted HS-proteoglycans (such as perlecan, collagen-XVIII, agrin) residing on basement membranes and/or periendothelial matrix may serve dual functions: although they may sequester tumor proangiogenic growth factors (e.g., FGF-2, VEGF, or PDGF) onto tumor vascular basement membrane and/or matrix HS chains, thereby facilitating tumor angiogenesis, the core proteins may also be proteolytically cleaved, with the release of angiostatic C-terminal fragments. Genetic alteration of the perlecan NH₂-terminal HS-attachment sites resulted in altered tumor and wound angiogenesis.¹¹⁵ Interestingly, perlecan-associated HS also may serve as a “rescue” mechanism during targeting of the tumor VEGF pathway. Specifically, an increase in perlecan and heparanase expression has been noted in regions of angiogenesis recovery in VEGFR-2 targeted hepatoblastoma xenografts.¹¹⁶ But then, proteolytic cleavage of the C-terminal domains of perlecan as well as collagen-XVIII, for example, leads to the release of the angiostatic fragments endorepellin and endostatin, respectively. These fragments may counter HS proteoglycan-mediated angiostimulatory processes through their actions on integrins ($\alpha2\beta1$ in the case of endorepellin¹¹⁷) or through downregulation of the VEGF signaling cascade and stimulation of thrombospondin-1 (in the case of endostatin¹¹⁸).

D. Other Forms of Pathologic Angiogenesis and Endothelial Heparan Sulfate

1. Lessons Learned from Proliferative Retinopathy and VEGF—Given the broad roles of HS in modulating the activity of multiple growth factors in tumor angiogenesis, in addition to the unique importance of HS proteoglycan core proteins in mediating distinct pathophysiologic steps in endothelial growth and migration, it is useful to consider how targeting HS might factor into the treatment of other angioproliferative diseases. In the last 10 years, anti-VEGF therapy, in the form of the humanized monoclonal antibody bevacizumab, has been FDA-approved for use in combination with chemotherapy in the treatment of colon cancer⁹⁰ and non-small cell lung cancer.⁹¹ Prior to this, elevated levels of VEGF-A were found in the aqueous and vitreous humor of human eyes in the setting of diabetic retinopathy as well as adult macular degeneration (AMD), particularly exudative (or “wet”) AMD.^{119,120} These findings established the basis for the intravitreal injectable treatments pegaptinib (Macugen), a pegylated oligonucleotide aptamer that binds VEGF165, and ranibizumab (Lucentis), a recombinant humanized Fab that binds all VEGF-A isoforms.^{121,122} Interestingly, these revolutionary AMD treatments not only slowed the loss of vision in such patients, but also led to an unexpected sustained gain of vision in a number of patients.¹¹⁹ Although one may consider a role for targeting HS in the light of limitations in efficacy as well as mechanisms of resistance to anti-VEGF therapy in tumors,⁹² targeting HS may also be a relevant topic in the consideration of current challenges in proliferative retinopathy, including combined therapies to improve duration of responses (e.g., beyond 24 months of anti-VEGF treatment in AMD) as well as minimize side effects.¹²³ Indeed, other proangiogenic pathways involving HS-binding growth factors appear to act in concert with VEGF-A during vascular progression in proliferative retinopathies, including AMD as well as diabetic retinopathy.^{124–126} Thus, the application of novel agents that either compete with

HS to simultaneously limit receptor activation by multiple growth factors (e.g., heparinoids or polysulfonated mimetics), or target HS biosynthesis (e.g., enzyme small-molecule inhibitors), may serve to limit levels of the anti-VEGF agent(s) while possibly prolonging action as well as limiting toxicity.

2. Novel Targeting of Angiogenesis in Other Chronic Inflammatory

Conditions: Targeting Heparan Sulfate?—In addition to cancer and proliferative vascular retinopathies, pathologic angiogenesis also occurs during inflammatory disorders, with particularly well-characterized examples occurring in rheumatoid arthritis and psoriasis. The HS proteoglycan core proteins syndecan-2, syndecan-3, and glypican-4 appear to be upregulated in the endothelium of inflamed synovial tissues in both rheumatoid and psoriatic arthritis (when compared with synovial samples from normal or osteoarthritic joints), wherein intense angiogenesis contributes to progression of rheumatic joint pathology.¹²⁷ Interestingly, the upregulation of HS on multiple endothelial proteoglycan core proteins in such disorders may not only contribute to angiogenesis through its coreceptor action on proangiogenic growth factor receptors, but also through the ability of HS to concentrate the chemokine CXCL12 in the rheumatoid hyperplastic lining layer and endothelium. It appears that synoviocyte-derived CXCL12 is immobilized and accumulates on endothelial cell HS, through which it promotes angiogenesis.¹²⁸ In this context, it may also be appealing to follow similar HS-targeting approaches as those proposed for proliferative vascular ocular disorders above, wherein HS itself may be targeted through competitive or small-molecule approaches. Given the simultaneous upregulation of a number of endothelial HS proteoglycan core proteins in rheumatoid arthropathy, targeting the (common) HS glycan chain may prove more promising than interference with specific core proteins.

E. Turning It Around—Therapeutic Angiogenesis and Endothelial Heparan Sulfate

1. The Actions of Proangiogenic Growth Factors in Cardiovascular Ischemic

Remodeling—Insights on the genetic importance of HS in mediating angiogenesis as a pathologic process allow us to better investigate a new direction: Can one facilitate angiogenesis as a therapeutic process in ischemic disorders, and how might one manipulate HS in a manner that promotes therapeutic angiogenesis? First, the state of hypoxia, especially chronic hypoxia, upregulates the expression of hypoxia-inducible transcription factors (HIF-1 and HIF-2), which in turn upregulates VEGF-A expression^{129,130} and vascular endothelial proliferation.¹²⁹ It is also interesting to note that cardiac ischemic models have shown that tissue upregulation of HS occurs as a result of the ischemic insult.^{131,132} Although it is possible that the latter facilitates the action of proangiogenic growth factors, the cellular distribution as well as fine structure of upregulated HS in this setting are poorly understood. Nevertheless, direct delivery of proangiogenic growth factors, including FGFs and VEGF, to the myocardium has been demonstrated in animal models of coronary ischemia (employing intracoronary adenoviral-mediated gene transfer as well as novel ultrasound-mediated myocardial plasmid delivery), with resultant postischemic stimulation of angiogenesis, and improvements in regional myocardial blood flow and function.^{133,134} It is possible that manipulating the production and structure of HS in the ischemic microenvironment (including that on the endothelium as well as ECM) may optimize the ability of HS to efficiently serve as a coreceptor such as growth factors, whether they are exogenously introduced or produced locally. This might also be achieved through targeted alterations in sulfatase and heparanase expression in ischemic tissues. Finally, upregulation or exogenous delivery of proangiogenic growth factors may have pathologic consequences, such as the Src-mediated vasogenic edema that results from VEGFR signaling pathway activation.¹³⁵ Thus, any strategy that augments specific growth factors, HS biosynthesis and/or fine structure, or both needs to be considered in light of not

only its proangiogenic potential, but also its net effect on capillary leak and ischemic tissue edema.

2. Chronic Wound Healing and Modifying Wound Granulation—Wound healing is characterized by an active phase of granulation-tissue proliferation, typically within the first few days following acute injury. This form of inflammatory remodeling is characterized by intense angiogenesis, and a variety of factors may limit or halt this proliferative response, leading to chronic wound progression, tissue necrosis, infection, and other complications.¹³⁶ Angiopoietins, VEGF, FGFs, and TGF- β are involved in stimulating this process, and their actions are coordinated with those of upregulated laminins in the ECM as well as integrins (principally beta-1 and alpha-v subunits), mediating the interactions between migrating cells and the matrix.¹³⁷ Augmenting the expression or structural features of HS, which is present on wound endothelium, vascular basement membranes, and the ECM, may improve granulation-tissue angiogenesis and wound healing. One interesting approach is the exogenous addition or transgenic expression of heparanase, with associated enhanced wound epithelialization as well as wound angiogenesis and vessel maturation.⁸¹ This may increase the availability of HS-bound growth factors, as occurs as a result of heparanase action during tumor angiogenesis, with subsequent stimulation of endothelial growth and angiogenesis. Genetic evidence points to matrix and basement membrane-associated HS expressed on perlecan as a particularly important form of HS presentation, contributing to granulation-associated angiogenesis.^{115,138} It is possible that targeted upregulation of perlecan in chronic nonhealing wounds might augment angiogenesis and the regeneration of healthy granulation tissue, promoting vital wound healing and closure. It is also possible that manipulating the expression or delivery of heparanase (and possibly sulfatases) in this context might modify the structure of endothelial and/or matrix HS in the wound environment in a manner that maximizes granulation-associated angiogenesis through optimal interactions of wound endothelium with growth factors and integrin-mediated interactions with matrix proteins.

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Fig. 1.

HS structure and biosynthesis: Each sugar residue is depicted by a geometric symbol. Binding sites for ligands are defined by the arrangement of sulfate groups (NS, 2S, 3S, 6S) and uronic acid epimers (GlcA and IdoA). In mammals, as many as 26 enzymes (italicized) participate in the formation of HS chains. *N*-deacetylase/*N*-sulfotransferase (Ndst) initiates sulfation in clustered sites along HS chains, and isoenzymes 1 and 2 (of four expressed in mammals), highlighted in *red*, are expressed in ECs. Through the action of other sulfating enzymes, further sulfation modifications take place around sites of clustered *N*-sulfation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this chapter.)

TABLE I

Phenotypes of Zebrafish or Mice that are Deficient in HS Biosynthetic Enzymes or Mice Deficient in HS-Binding Angiogenic Factor or Their Receptors

Gene	Phenotype
<i>MC-Ext-1</i> ^{-/-} mice	Embryonic lethality associated with vascular patterning defects, edema, and hemorrhages during late gestation. MC recruitment in the skin with impaired vessel stability and variable diameters. ²⁶
<i>Ndst-1</i> ^{-/-} mice	Neonatal lethal, pulmonary atelectasis, cyanosis ²⁷⁻²⁹ ; pericyte detachment and delayed migration due to impaired PDGF-B signaling and disruption of directed cell migration. ³⁰
<i>EC-Ndst-1</i> ^{-/-} mice	Impaired inflammatory response and tumor angiogenesis. ^{31,32}
<i>Hepi</i> ^{-/-} mice	Die shortly after birth with multiple developmental defects, such as skeletal malformations and kidney agenesis, ³³ and transient delay of MC recruitment during embryonic vascular development. ³⁰
Hs6st-2 morphant	Abnormalities in the branching morphogenesis of the caudal vein during embryonic development. Hs6st-2 interacts synergistically with VEGF-A in angiogenesis in vivo. ³⁴
<i>Hs6st-1</i> ^{-/-} mice	Most die between E15.5 and perinatal stage. Approximately 50% reduction in microvessel density in the labyrinthine zone of placenta with a modest reduction in VEGF-A mRNA and protein. ^{35,36}
<i>Hs3st-1</i> ^{-/-} mice	No obvious procoagulant phenotype, background-specific lethality and intrauterine growth retardation, severely compromised fertility and disrupted maternal-fetal circulation in. ^{37,38}
VEGF120/120 mice	Impaired postnatal myocardial angiogenesis, cardiomyopathy and lethal cardiac failure, reduced vascular branching complexity and increased vascular caliber. ³⁹⁻⁴¹
<i>VEGFR1 (flt-1)</i> ^{-/-} mice	E8.5-9.5 Embryonic lethality exhibiting overgrowth of ECs and disorganized vasculature. ⁴²
<i>VEGFR2 (flk-1)</i> ^{-/-} mice	Homozygous: E8.5-9.5 Embryonic lethality, severely impaired hematopoietic and ECs; heterozygous appear normal. ⁴³
<i>PDGF-Bret</i> ^{-/-} mice	Vascular defect with pericyte detachment. ⁴⁴
<i>PDGF-BB</i> ^{-/-} mice	Embryonic lethality. MC loss which results in vascular defects including endothelial hyperplasia, capillary dilation, microaneurysms, vascular leakage, and hemorrhaging. ^{45,46}
<i>PDGFR-β</i> ^{-/-} mice	Similar to the PDGF-BB ^{-/-} mice.
TGFRII-β ^{-/-} mice	Defective vascular development associated with reduced MC recruitment and impaired vessel stability. ⁴⁸