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Mice deficient in heparan sulfate 3-*O*-sulfotransferase-1: Normal hemostasis with unexpected perinatal phenotypes

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Heparan sulfate that contains antithrombin binding sites is designated as anticoagulant heparan sulfate (HS^{act}) since, *in vitro*, it dramatically enhances the neutralization of coagulation proteases by antithrombin. Endothelial cell production of HS^{act} is controlled by the *Hs3st1* gene, which encodes the rate limiting enzyme—heparan sulfate 3-*O*-sulfotransferase-1 (Hs3st1). It has long been proposed that levels of endothelial HS^{act} may tightly regulate hemostatic tone. This potential *in vivo* role of HS^{act} was assessed by generating *Hs3st1*$^{-/-}$ knockout mice. *Hs3st1*$^{-/-}$ and *Hs3st1*$^{+/+}$ mice were evaluated with a variety of methods, capable of detecting altered hemostatic tone. However, both genotypes were indistinguishable. Instead, *Hs3st1*$^{-/-}$ mice exhibited lethality on a specific genetic background and also showed intrauterine growth retardation. Neither phenotypes result from a gross coagulopathy. So although this enzyme produces the majority of tissue HS^{act}, *Hs3st1*$^{-/-}$ mice do not show an obvious procoagulant phenotype. These results suggest that the bulk of HS^{act} is not essential for normal hemostasis and that hemostatic tone is not tightly regulated by total levels of HS^{act}. Moreover, the unanticipated non-thrombotic phenotypes suggest structure(s) derived from this enzyme might serve additional/alternative biologic roles.
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The potential *in vivo* role of HS^{act}

We have generated mice deficient in heparan sulfate 3-*O*-sulfotransferase (Hs3st1) to evaluate the *in vivo* role of "anticoagulant heparan sulfate" (HS^{act}). HS^{act} contains a specific pentasaccharide (Figure 1) that was originally identified as the structure [1–7] conveying anticoagulant activity to pharmaceutical heparin through selective interactions with antithrombin. In the absence of heparin, antithrombin slowly neutralizes proteases of the coagulation cascade by forming 1:1 protease-antithrombin covalent complexes. Conversely, when antithrombin binds heparin, the rate of neutralization is catalyzed by ~1000-fold. Both binding and activation of antithrombin absolutely require the central 3-*O*-sulfate moiety [5,6].

Given such a dramatic biochemical effect, it has long been postulated that endogenous heparan sulfate proteoglycans (HSPGs) might similarly enhance antithrombin activity to regulate blood coagulation [8]. However, therapeutic heparin acts in the blood, which does not normally contain free endogenous HSPGs. Instead, core proteins bearing HS^{act} are "tethered" to vascular endothelial cells. Such a location might allow a hemostatic role—as the perfusion of purified thrombin and antithrombin into the hind limbs of rodents leads to an elevated rate of thrombin-antithrombin complex formation (for both normal and mast cell-deficient animals) [9,10].

So how might vascular HS^{act} regulate coagulation? HS^{act} is much less abundant than antithrombin, so HS^{act} should only activate a small fraction of total plasma antithrombin. Human genetics supports this notion. Patients that express antithrombin

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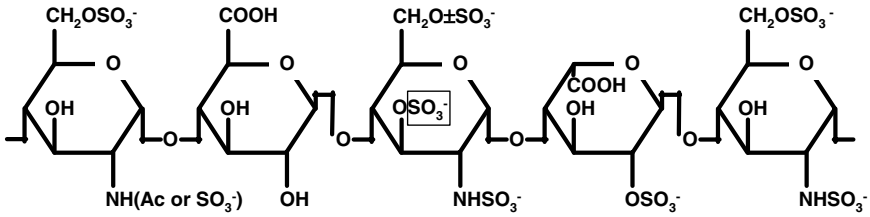

Figure 1. The antithrombin-binding site of heparan sulfate. Although several moieties make contact with antithrombin, the central 3-*O*-sulfate (boxed) is absolutely essential for binding and catalysis.

variants with reduced affinity for heparin (type II heparin binding-site) typically develop thrombosis only if both antithrombin genes are affected [reviewed by 11,12]. Since heterozygotes are usually unaffected, it appears only a subfraction of normal antithrombin molecules is essential for HS^{act} function. Potentially, the level of endothelial HS^{act} defines how much antithrombin is activated and thereby regulates hemostatic tone. Such a mechanism is supported by two factors. First, the plasma antithrombin concentration ($\sim$5 μM) appears saturating, i.e. this level greatly exceeds the dissociation constant of antithrombin for HS^{act} ($\sim$15 nM) [13,14]. Saturation of HS^{act} by antithrombin implies this anticoagulant system is functioning continuously, which is consistent with the dynamic nature of hemostasis. Hemostatic tone being continuously established as the net balance between ongoing antagonistic processes (procoagulant versus anticoagulant and fibrinolytic) [reviewed by 12,15]. Second, regulation of HS^{act} levels by endothelial cells occurs. Only a small subpopulation of HSPGs actually bears HS^{act} and this level of HS^{act} varies between endothelial cells isolated from different vascular beds [16–18]. Moreover, analyses of mutant cell lines show that HS^{act} levels can be selectively altered without influencing the bulk of HS [19–21]. In summary, several lines of evidence suggest that endothelial cell regulation of HS^{act} levels might control antithrombin activation and thereby contribute to hemostatic balance.

However, an *in vivo* anticoagulant role for HS^{act} has not been conclusively demonstrated. In fact, the probing of blood vessels with ^{125}I-antithrombin reveals >95% of HS^{act} are localized to the abluminal endothelial surface; thereby lacking direct contact with blood [22]. This paradoxic location challenges the potential for HS^{act} to catalyze the anticoagulant activity of antithrombin; accordingly, we have endeavored to evaluate the hemostatic role of HS^{act}.

Experimental approach—generating mice deficient in Hs3st1

To test whether HS^{act} levels modulate hemostatic tone, we generated mice with reduced levels of HS^{act}. Reducing HS^{act} can not easily be accomplished through manipulating core proteins, as multiple core types can bear HS^{act} [16,23,24]. Furthermore, a single core can bear either HS^{act} or HS that lacks the critical pentasaccharide [19]. Instead, we choose to eliminate the sulfotransferase Hs3st1. This enzyme preferentially creates the above pentasaccharide from a precursor by adding the 3-*O*-sulfate that is absolutely essential for antithrombin binding and activation [7,25,26]. In many cell-types this enzyme is limiting and thereby defines cellular production of HS^{act} [27]. Moreover 3-*O*-sulfates typically comprise less than 1% of HS sulfation [19,21], so elimination of this sulfotransferase should produce a selective effect on HS^{act} without perturbing bulk HS production. It is important to note that four additional Hs3st isoforms have been isolated [28]. Compared to Hs3st1, these isoforms have dramatically distinct substrate preferences, presumably to regulate distinct biologic properties of HS [28,29]. These additional isoforms can generate some HS^{act}, but at about 250-fold less efficiency than Hs3st1 [unpublished data and 30]. Clearly, Hs3st1 is likely to be the dominant isoform regulating *in vivo* HS^{act} production. Thus, to evaluate whether HS^{act} levels influence coagulation, we generated mice deficient in Hs3st1 [31].

The Hs3st1 enzyme is encoded by the *Hs3st1* gene with the entire coding region being encompassed within exon 8. A replacement vector was designed to eliminate exon 8 so as to generate mice lacking all Hs3st1 coding sequences. Embryonic stem cells (from a 129/Sv substrain) that had undergone appropriate recombination were injected into blastocysts to generate chimeric mice, which were bred to C57BL/6J mice. Interbreeding of heterozygotes resulted in viable mice with comparable recovery of nulls and the wild-types (N1 of Table 1). That $Hs3st1^{-/-}$ mice were viable was initially confusing, as mice completely deficient for antithrombin die *in utero* [32]. However, $Hs3st1^{-/-}$ mice were clearly devoid of Hs3st1 enzyme [31], as revealed by examining tissue homogenates and plasma for HS^{act} conversion activity–an assay that measures Hs3st1-dependent formation of antithrombin-binding sites within HS [27].

Reduced HS^{act} levels do not produce an obvious procoagulant state

HS has been isolated from a variety of tissues to test the effect of *Hs3st1* disruption on HS^{act} levels [31]. In keeping with the rarity of 3-*O*-sulfates, total HS tissue levels are comparable between $Hs3st1^{-/-}$ and $Hs3st1^{+/+}$ mice. For most $Hs3st1^{-/-}$ tissues examined, the *in vitro* anti-Xa activity of HS was reduced by 75–98%, compared to $Hs3st1^{+/+}$ material (Figure 2a). Tissue proteoglycans also showed a comparable reduction in ^{125}I-antithrombin-binding sites [31]. The minor

Table 1. Genotypes of offspring from heterozygous parents (*Hs3st1*$^{+/-}$ × *Hs3st1*$^{+/-}$)

Parental background[1]	*Offspring C57BL/6 content (%)*	*Offspring Hs3st1 genotype*			*+/+:−/− ratio*	*Loss of Hs3st1*$^{-/-}$[2]	*P*[3]	*Age*[4]
		+/+	*+/−*	*−/−*				
N1	∼50.0	35	106	35	1:1	–		P21
N3	∼87.5	21	45	5	1:0.24	76%	<0.003	P21
N4	∼93.8	16	13	1	1:0.06	94%	<0.001	P21
N6	∼98.4	10	17	12	1:1	–		E18.5
N6	∼98.4	18	26	11	∼1:1	39%		P0 + P1
N7	∼99.2	14	8	2	1:0.14	86%	<0.001	P14
$N8_{male}$ X 129S4/ $SVJae_{female}$	50.0	25	56	22	1:1	–		P21

[1]N indicates number of successive backcrosses against C57BL/6 to generate the parental *Hs3st1*$^{+/-}$. [2]Loss expressed relative to *Hs3st1*$^{+/+}$. [3]Probability of a non-Mendelian outcome determined by Chi-squared test. [4]Age at which tissue was collected for genotyping. *P* indicates days post birth whereas E indicates embryonic days post conception. Modified from [31].

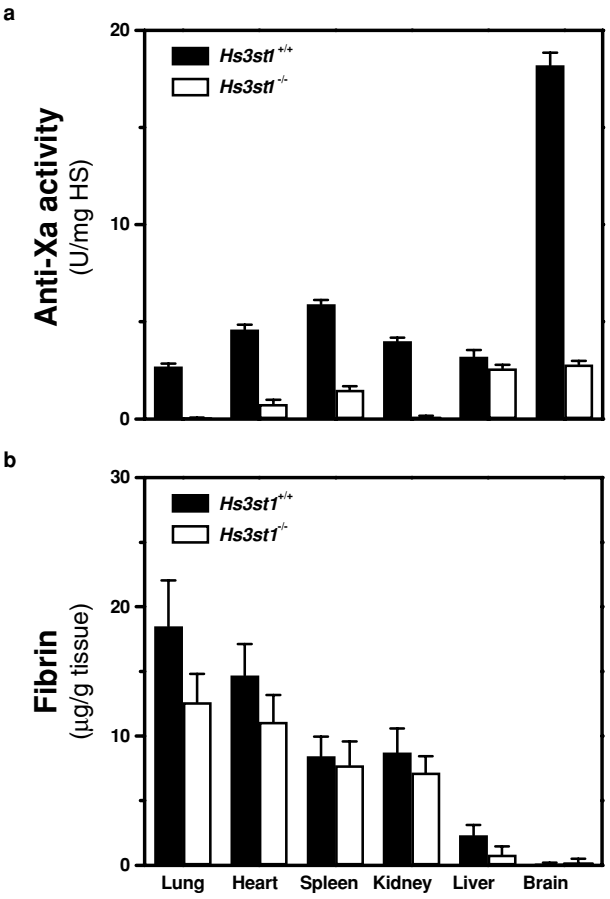

Figure 2. *Hs3st1*$^{-/-}$ mice have reduced tissue HS^{act} levels but normal fibrin accumulation. (a) Heparan sulfate was purified from tissues and *in vitro* anti-Xa activity was determined as HS catalysis of factor Xa neutralization by antithrombin ($n = 3$ littermates per group). (b) Western blots of tissue extracts were probed with a fibrin specific antibody to determine tissue fibrin accumulation. ($n = 10$ littermates per group). Results are mean ±S.E.M. Taken from [31].

residual antithrombin-binding sites and anti-Xa activity suggests other Hs3st isoforms can contribute to HS^{act} production. However for most tissues, Hs3st1 is clearly the predominant isoform that produces the vast majority of antithrombin-binding sites.

If HS^{act} contributes to ongoing hemostatic tone, then such large reductions in HS^{act} should generate a procoagulant state. Consequently, we examined basal tissue accumulation of fibrin (Figure 2b), which is an extremely sensitive index of microvascular hemostatic balance [33–35]. Surprisingly, tissue fibrin levels for *Hs3st1*$^{-/-}$ mice were indistinguishable from *Hs3st1*$^{+/+}$ littermates, even in organs with extremely low residual levels of anti-Xa activity (e.g. *Hs3st1*$^{-/-}$ lung and kidney, Figure 2a). Thus, a basal procoagulant state was not detected.

Mice were also subjected to a procoagulant challenge–overnight hypoxia (8% O_2), in an attempt to uncover a latent procoagulant condition. Prolonged hypoxia elevates tissue factor levels in monocytes, macrophages and pulmonary vascular endothelial cells, which elevates fibrin accumulation in the lung [33,36]. However *Hs3st1*$^{-/-}$ mice exhibited wild-type elevations in lung fibrin levels [31] despite the large HS^{act} reduction in *Hs3st1*$^{-/-}$ lung (Figure 2a). So a strong thrombotic challenge also failed to reveal a microvascular procoagulant state in *Hs3st1*$^{-/-}$ mice.

Since most of HS^{act} occurs in subendothelial matrix [22], an anticoagulant role might only occur following endothelial denudation, which would allow direct contact of the blood and subendothelial matrix. Probing with ^{125}I-antithrombin has shown that *Hs3st1*$^{-/-}$ blood vessels are substantially depleted in HS^{act} [31], which might make vessels prone to rapid thrombosis when endothelial injury exposes the subendothelial matrix. This hypothesis was tested with an arterial injury assay capable of detecting a procoagulant state [37–39]. Common carotid arteries were chemically injured with $FeCl_3$, which results in focal endothelial denudation and rapid growth of a platelet-rich thrombus that ultimately occludes blood flow. However,

Hs3st1$^{-/-}$ and *Hs3st1*$^{+/+}$ mice exhibited indistinguishable thrombus morphology, time to generate a complete occlusion, and post-injury levels of thrombin-antithrombin complexes [31]. Hence, the profound reduction in subendothelial matrix HS^{act} in *Hs3st1*$^{-/-}$ mice did not expedite occlusive thrombosis nor alter plasma levels of thrombin-antithrombin complexes.

The above data suggest that hemostatic tone is not tightly linked to HS^{act} levels. As a reciprocal approach, we have employed retroviral vectors that over-express the Hs3st1 cDNA and thereby test whether elevated HS^{act} levels could enhance antithrombin neutralization of α-thrombin/factor Xa (antithrombin activity) [31]. We transduced a nonendothelial cell line (CHO cells) and primary mouse cardiac microvascular endothelial cells. CHO cells lack endogenous Hs3st1 and do not synthesize HS^{act}. Consequently, CHO monolayers do not catalyze antithrombin activity. Conversely, cardiac microvascular endothelial cells express Hs3st1, synthesize HS^{act}, and catalyze antithrombin neutralization of proteases. In CHO cells, over-expression of Hs3st1 induced both HS^{act} synthesis and the ability of monolayers to catalyze antithrombin activity. However with the endothelial cells, over-expression of this enzyme augmented HS^{act} synthesis 3-fold but failed to enhance the cell surface's pre-existing high catalysis of antithrombin activity [31]. This *in vitro* evaluation shows that in endothelial cells, catalysis of thrombin-antithrombin complex formation may not be tightly linked to HS^{act} levels.

Perinatal phenotypes do not involve a gross procoagulant state

The Hs3st1-deficient mice are further remarkable as they develop several unanticipated abnormalities. These include spontaneous eye degeneration, aberrant cardiovascular response to anesthesia, reduced male and female fertility, postnatal lethality and intrauterine growth retardation. The perinatal phenotypes are of particular importance as such events can arise when defects in anticoagulant tone, such as antithrombin deficiency, lead to enhanced clotting [32,40]. Consequently, we have endeavored to test whether a procoagulant state might be responsible for perinatal traits of *Hs3st1*$^{-/-}$ mice. These phenotypes were detected while manipulating mouse genetic background. Knockout mice were initially generated on a mixed genetic background (C57BL/6 X 129S4/SvJae). To generate animals on a pure genetic background, the knockout allele was successively bred through C57BL/6 mice and the resulting *Hs3st1*$^{+/-}$ mice were interbred. As the C57BL/6 content increased, the recovery of *Hs3st1*$^{-/-}$ weanlings diminished (Table 1). Recovery of *Hs3st1*$^{-/-}$ weanlings leveled out at $\sim$10% of *Hs3st1*$^{+/+}$ numbers after 4 backcrosses (equal recovery at N4 and N7, Table 1). Conversely, *Hs3st1*$^{-/-}$ lethality is completely rescued by interbreeding C57BL/6 and 129S4/SvJae mice to recreate the original hybrid genetic background (Table 1, N8 male X 129S4/SvJae female) [31]. Thus, the *Hs3st1*$^{-/-}$ genotype is lethal when placed on a C57BL/6 background.

Further characterizations were conducted to determine whether *Hs3st1*$^{-/-}$ mice mimic antithrombin deficient (*ATIII*$^{-/-}$) mice, which show intrauterine lethality [32]. Stage of death was assessed by determining perinatal genotypes one day before birth, on the day of birth and one day post birth (E18.5, P0 and P1 respectively). *Hs3st1*$^{-/-}$ mice showed normal viability at E18.5 (Table 1, analysis at N6) in contrast to *ATIII*$^{-/-}$ mice, which showed complete lethality two days earlier (E16.5). Recovery of *Hs3st1*$^{-/-}$ pups was only slightly reduced at P0/P1 (Table 1) and lethality was probably complete by P2-P3 [31]. So *Hs3st1*$^{-/-}$ mice exhibited postnatal lethality, which contrasts to the intrauterine lethality of *ATIII*$^{-/-}$ mice. Moreover, *Hs3st1*$^{-/-}$ pups do not exhibit bruising nor subcutaneous hemorrhages. Such signs are hallmarks of purpura fulminans [40]–a hypercoagulable state in humans that can lead to postnatal lethality. Furthermore, histological surveys of E18.5 and P0 animals failed to reveal thrombosis or hemorrhage. Most importantly, myocardial and hepatic tissues lacked focal thrombosis and degeneration, which invariably occur in late stage *ATIII*$^{-/-}$ embryos [32]. Thus, *Hs3st1*$^{-/-}$ lethality is very distinct from *ATIII*$^{-/-}$ lethality and does not appear to result from a gross coagulopathy.

The cause of the lethality is presently unclear, since *Hs3st1*$^{-/-}$ newborns have unlabored breathing, ingest milk, produce and excrete urine, and exhibit normal startle reflexes to noise and motion. Moreover, histologic surveys failed to detect any anatomic malformations [31]. However, *Hs3st1* genotype does influence embryonic mass in a dose dependent fashion with *Hs3st1*$^{+/-}$ and *Hs3st1*$^{-/-}$ E18.5 embryos respectively being 8% and 20% underweight, compared to wild-types [31]. Thus, Hs3st1 deficiency causes intrauterine growth retardation. It is intriguing that heterozygotes show both a partial degree of intrauterine growth retardation and a partial extent of lethality (Table 1). Potentially, intrauterine growth retardation in combination with the C57BL/6 background causes lethality. Although the extent of intrauterine growth retardation is "small" it is important to note that C57BL/6 newborns are already substantially smaller than newborn C57BL/6 X 129S4/SVJae hybrid mice. So on a C57BL/6 background the additional growth retardation from Hs3st1 deficiency might be just sufficient to tip the balance and compromise viability. Maternal factors might additionally play a role. Culling of the litter by infanticide can occur during the first postnatal week. In this "quality control" process, mothers preferentially eliminate the smallest pups [41]. While such a scenario is consistent with the timing of *Hs3st1*$^{-/-}$ lethality, alternate explanations cannot be ruled out.

Intrauterine growth retardation can result from procoagulant states that induce placental vascular insufficiency [42,43]. However, three factors indicate this situation does not occur in *Hs3st1*$^{-/-}$ mice. First, placental insufficiency typically leads to elevated embryo/placental weight ratios [42,43]; but *Hs3st1*$^{-/-}$ and *Hs3st1*$^{+/+}$ mice show comparable ratios [31]. Second,

intrauterine growth retardation from placental insufficiency is usually "asymmetric", with preferential sparing of head growth [44–47]; however, *Hs3st1*$^{-/-}$ embryos show "symmetric" intrauterine growth retardation with comparable reductions in head and body mass [31]. Third, *Hs3st1*$^{-/-}$ placentae are histologically normal [31] and lack the key features of thrombotic vascular insufficiency (thrombi and avascular villi) [48]. Thus, the intrauterine growth retardation of *Hs3st1*$^{-/-}$ embryos appears to be distinct from placental vascular insufficiency. Given that *Hs3st1*$^{-/-}$ embryos also lack thrombosis, the combined data suggest that an overt procoagulant state is not responsible for the intrauterine growth retardation of *Hs3st1*$^{-/-}$ mice.

Does HS^{act} serve an anticoagulant role?

The above data show that *Hs3st1*$^{-/-}$ mice have substantially reduced levels of HS^{act}. We have evaluated these animals by techniques capable of detecting altered hemostatic balance [32–34, 37–39, 49–51]. Surprisingly, these approaches show that these mice have normal hemostasis. Moreover, the postnatal lethality and intrauterine growth retardation of *Hs3st1*$^{-/-}$ mice do not result from a gross procoagulant state. In particular the presentation of *Hs3st1*$^{-/-}$ lethality is very distinct from that of *ATIII*$^{-/-}$ mice, even though HS^{act} is proposed to be critical for antithrombin activity. Since large reductions in HS^{act} do not induce a procoagulant state, it appears that hemostatic balance may not be tightly linked to HS^{act} levels. This assertion is supported by the over-expression of Hs3st1 in endothelial cells, which elevates HS^{act} levels but fails to augment the preexisting high cell surface catalysis of thrombin-antithrombin complex formation.

However, these data do not rule out an anticoagulant role for HS^{act}, as suggested by recently generated knockin mice that express a mutant antithrombin form incapable of heparin binding and catalysis. Over the first year of life such mice spontaneously die from gross thrombosis .[Dewerchin, personal communication and 52]. So why might *Hs3st1*$^{-/-}$ mice lack a procoagulant phenotype? First, procoagulant knock-out mice show enhanced fibrin accumulation in a distinct spectrum of tissues [33,34,39,49]. The tissues examined in our present study have previously been sufficient to demonstrate a procoagulant state in such knockout mice. However, Hs3st1-generated HS^{act} potentially might serve an anticoagulant role in only a minor tissue not yet examined. Second, a hemodynamic perturbation might be masked by compensatory mechanisms. Unmasking of an overt procoagulant effect might require adding a second genetic defect to *Hs3st1*$^{-/-}$ mice. For example combining the deficiencies for tissue plasminogen activator and thrombomodulin leads to an extreme hypercoagulable state with myocardial necrosis and depressed cardiac function [34].

Third, Hs3st1-deficient mice maintain a small residual amount of HS^{act}. On the one hand, low levels of HS^{act} might be sufficient for normal hemostatic balance. On the other hand, only a specific subpopulation of HS^{act} may be involved in hemostasis. For example, HS^{act} on the endothelial luminal (apical) surface is relatively minimal, but such localization might still be essential for anticoagulant activity. Potentially, luminal HS^{act} may be unaffected in Hs3st1 deficiency. So in terms of hemostatic balance the critical factor may not be levels of total HS^{act} but rather the amount of HS^{act} at the luminal surface. Residual HS^{act} is likely produced by another Hs3st isoform. Conceivably, this alternate isoform might only modify heparan sulfate attached to core protein(s) that selectively localize to the apical surface. Thus HS^{act} that regulates clotting might be derived from an alternate Hs3st isoform, and not Hs3st1. Discriminating between these possibilities will require the identification of the isoform(s) responsible for the HS^{act} that remains in *Hs3st1*$^{-/-}$ mice.

Potential candidates include Hs3st-3$_A$ and Hs3st-3$_B$, which are expressed in virtually all tissues [28]; however, these isoforms are not efficient at generating HS^{act} [28,30]. Instead, the Hs3st-3 forms preferentially produce the gD binding site, a distinct structure that enables cellular entry by herpes simplex virus I. Alternatively, we have recently identified the final gene family members, Hs3st-5 and Hs3st-6. The former is most homologous to Hs3st1, in terms of both genomic organization and protein structure. Intriguingly, Hs3st-5 can generate both antithrombin-binding sites and gD-binding sites. Compared to all other candidates, Hs3st-5 is far more efficient at producing antithrombin-binding sites. Moreover Hs3st-5 shows relatively low ability to produce gD binding sites, compared to Hs3st-3 forms (Rhodes, HajMohammadi, Seeberger, McNeely, Spear, and Shworak; unpublished data). So from the perspective of biochemical specificity, Hs3st-5 appears the preferred candidate for making residual HS^{act}. Conclusive identification of the responsible isoform(s) will additionally require characterizations of isoform expression sites and levels in normal and Hs3st1 deficient animals.

Does HS^{act} serve a non hemostatic role(s)?

Instead of an anticipated procoagulant state, *Hs3st1*$^{-/-}$ mice exhibit several unexpected phenotypes including neonatal lethality and intrauterine growth retardation. The lethality might be secondary to intrauterine growth retardation; however, the critical time/developmental stage for required Hs3st1 expression has yet to be determined. Although such perinatal events can be caused by a gross procoagulant state, such dysfunction does not occur in Hs3st1-deficient animals. Consequently, the unexpected phenotypes raise the possibility that HS^{act} participates in a novel biologic function(s). Indeed, recent analyses of *Drosophila* deficient for the isologous enzyme, dHs3st-A, suggest a critical role in developmental signaling pathways [H. Nakato, personal communication]. Novel functions might even occur outside of the vascular system, as Hs3st1-derived HS^{act} is produced in several nonendothelial cell types, including ovarian granulosa cells [53] and many epithelial cell types [A. de Agostini, personal communication]. However, Hs3st1 is also capable of generating a distinct array of structures that do not

bind antithrombin [7,26]. Thus, unexpected phenotypes may reflect functions of either HS^{act} or alternative Hs3st1-generated structures. Differentiating between these possibilities will ultimately require complementation experiments with Hs3st1 forms that, hypothetically, have been engineered to selectively produce either HS^{act} or alternative structures. However, such experiments must await the identification of the involved developmental stage and cell-type as well as the engineering of Hs3st1 forms to create greater sequence selectivity.

Summary

Mice deficient in Hs3st1 show large reductions in HS^{act} but lack an anticipated procoagulant phenotype. This surprising observation suggests that the bulk of HS^{act} is not essential for normal hemostasis and that hemostatic tone may not be tightly linked to total HS^{act} levels. More extensive genetic manipulations of *Hs3st1*$^{-/-}$ mice will be required for further evaluation of HS^{act} as a natural anticoagulant. The unanticipated phenotypes that develop in *Hs3st1*$^{-/-}$ mice raise the possibility that Hs3st1-generated structures may participate in additional or alternative biologic processes. Like opening Pandora's box, the generation of *Hs3st1*$^{-/-}$ mice has had unforeseen consequences, which eventually should provide a fuller understanding of how biosynthetic processes control the functional diversity of heparan sulfate.

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