Normal levels of anticoagulant heparan sulfate are not essential for normal hemostasis

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Endothelial cell production of anticoagulant heparan sulfate (HS\textsuperscript{act}) is controlled by the Hs3st1 gene, which encodes the rate-limiting enzyme heparan sulfate 3-O-sulfotransferase-1 (3-OST-1). In vitro, HS\textsuperscript{act} dramatically enhances the neutralization of coagulation proteases by antithrombin. The in vivo role of HS\textsuperscript{act} was evaluated by generating Hs3st1–/– knockout mice. Hs3st1–/– animals were devoid of 3-OST-1 enzyme activity in plasma and tissue extracts. Nulls showed dramatic reductions in tissue levels of HS\textsuperscript{act} but maintained wild-type levels of tissue fibrin accumulation under both normoxic and hypoxic conditions. Given that vascular HS\textsuperscript{act} predominantly occurs in the subendothelial matrix, mice were subjected to a carotid artery injury assay in which ferric chloride administration induces de-endothelialization and occlusive thrombosis. Hs3st1–/– and Hs3st1+/– mice yielded indistinguishable occlusion times and comparable levels of thrombin•antithrombin complexes. Thus, Hs3st1–/– mice did not show an obvious procoagulant phenotype. Instead, Hs3st1–/– mice exhibited genetic background–specific lethality and intraterine growth retardation, without evidence of a gross coagulopathy. Our results demonstrate that the 3-OST-1 enzyme produces the majority of tissue HS\textsuperscript{act}. Surprisingly, this bulk of HS\textsuperscript{act} is not essential for normal hemostasis in mice. Instead, 3-OST-1–deficient mice exhibited unanticipated phenotypes suggesting that HS\textsuperscript{act} or additional 3-OST-1–derived structures may serve alternate biologic roles.


Introduction
Hemostatic tone is dynamically established as the net balance between ongoing procoagulant versus anticoagulant and fibrinolytic processes. Antithrombin (AT) is a major anticoagulant that slowly neutralizes proteases of the coagulation cascade through the formation of 1:1 enzyme•AT complexes. The rate of neutralization is dramatically enhanced by heparin, a variant of heparan sulfate (HS) from mast cells. Damus et al. (1) hypothesized that HS proteoglycans (HSPGs) on the endothelial cell surface might similarly accelerate AT activity and thereby contribute to the nonthrombogenic properties of blood vessels. Indeed, the perfusion of purified thrombin (T) and AT into the hindlimbs of rodents led to an elevated rate of T•AT–complex formation that was HS dependent. Endothelial cells produce only a small subpopulation of anticoagulant HS (HS\textsuperscript{act}) that binds AT and accelerates in vitro T•AT complex generation (reviewed in refs. 2 and 3). This property distinguishes HS\textsuperscript{act} from the bulk of nonanticoagulant HS (HS\textsuperscript{inact}), which lacks in vitro anticoagulant activity. It is presently unclear, however, whether HS\textsuperscript{act} is a major physiologic modulator of hemostasis.

For major hemostatic regulators, changes in the activity level can result in a hypercoagulable state (reviewed in refs. 3–5). For example, mutations that reduce the level of AT activity primarily predispose patients to venous thrombosis. Complete AT deficiency appears incompatible with human life and in mice causes intrauterine death from an extreme hypercoagulable state, consumptive coagulopathy (6, 7). Yet, the

See the related Commentary beginning on page 952.
The regulation of HS<sub>act</sub> production has been elucidated over the past decade. Core proteins appear to exert minimal influence, because a single core can bear either HS<sub>act</sub> or HS<sub>inact</sub> (9). Instead, HS<sub>act</sub> results from a discrete biosynthetic pathway regulated by a limiting biosynthetic factor (9, 10). Establishment of conditions for cell-free synthesis of HS<sub>act</sub> revealed a limiting activity that modifies only a portion of potential precursors, thereby defining cellular production of HS<sub>act</sub> (18). The critical enzyme was purified, cloned, and identified as the long-sought heparan sulfate 3-O-sulfotransferase-1 (3-OST-1) (19, 20), which preferentially modifies selected HS structures to create the above pentasaccharide. It also creates a limited range of 3-O-sulfated structures that do not bind AT (21), but the biologic relevance of these structures is unknown. To date, 3-OST-1 has only been implicated in regulating hemostatic tone. Four additional 3-OST isoforms have been isolated, but these isoforms have dramatically distinct substrate preferences; therefore, they may regulate distinct biologic properties of HS (22–24). Some of these isoforms can generate HS<sub>act</sub>, but at about 250-fold lower efficiency than 3-OST-1 (25). Thus, 3-OST-1 appears to be the dominant isof orm regulating in vivo HS<sub>act</sub> production. Moreover, selective regulation stems from the enzymatic specificity of 3-OST-1 and the paucity of 3-O-sulfates within HS.

Here, we investigated whether HS<sub>act</sub> levels influence hemostatic balance by generating <i>Hs3st1<sup>–/–</sup></i> mice that are deficient in 3-OST-1 enzyme and show large reductions in HS<sub>act</sub>.

**Methods**

**Generation of <i>Hs3st1<sup>–/–</sup></i> mice**

**Isolation of the <i>Hs3st1</i> coding region.** An arrayed P1 library of 129P2/OlaHsd genomic DNA was PCR screened (Genome Systems Inc., St. Louis, Missouri, USA) with two different oligonucleotide sets designed to amplify 5′ and 3′ untranslated region sequences. Primers 5′-dATGGCAACTGGAGATACTCATGT and 5′-dCTCCGGTGTCCTCTCCTC amplifying nucleotides 219–467, whereas 5′-dTTCTGTACAGTATTAGATTCACAGT with 5′-dGCTATTTTTGGATGGGCAAGCGT amplifying nucleotides 1383–1617 from the mouse 3-OST-1 cDNA sequence (20). Three independent overlapping genomic clones were recovered, exons were mapped by Southern blotting, and the coding region was verified by DNA sequencing. This analysis revealed that exon 8 contained the entire coding region. A detailed description of this gene shall be presented elsewhere.

**Gene targeting.** A targeting vector (Figure 1a) was constructed from pMCIDT-A (a gift from Helene Baribault, Burnham Institute, La Jolla, California, USA) and pBluescript SK(−) (Stratagene, La Jolla, California, USA) with two different oligonucleotide sets designed to amplify 5′ and 3′ untranslated region sequences. Primers 5′-dATGGCAACTGGAGATACTCATGT and 5′-dCTCCGGTGTCCTCTCCTC amplifying nucleotides 219–467, whereas 5′-dTTCTGTACAGTATTAGATTCACAGT with 5′-dGCTATTTTTGGATGGGCAAGCGT amplifying nucleotides 1383–1617 from the mouse 3-OST-1 cDNA sequence (20). Three independent overlapping genomic clones were recovered, exons were mapped by Southern blotting, and the coding region was verified by DNA sequencing. This analysis revealed that exon 8 contained the entire coding region. A detailed description of this gene shall be presented elsewhere.
Targeted D3 ES cells were generated and injected into C57BL/6 blastocysts as described previously (26).

**Genotyping.** Initially, genotyping was conducted by Southern blot analysis. BamHI-digested genomic DNA from ES cell clones or mouse tails was hybridized to external probes generated by PCR of cloned genomic sequences. A 120-bp 5′ probe was obtained with 5′-dGGATCCCTCGCCTGGTCTTAC and 5′-dTTCTGTACAGTATTAGATTCACAGT, whereas a 521-bp 3′ probe was amplified with 5′-dCTCCTGAGTCACCTACACTGAG and 5′-dGGATCCAGGACTAACTGACTTT. Subsequently, genotyping was conducted by heteroduplex PCR. The wild-type allele generates a 235-bp fragment with 5′-dGCCAGCGGGGCT-TGGATTGGAGGCAGGT, whereas, the knockout allele produces a 380-bp product with 5′-dGGATCCAGGACTAACTGACTTT.

**Breeding 3-OST-1–deficient mice.** Chimeras were bred to C57BL/6 mice, and heterozygous progeny were interbred to create F2 individuals of approximately 50:50 mixed genetic background, which were used for all characterizations unless stated otherwise. Chimeras were also bred to 129S4/SvJae mice to place the knock-out allele on an incipient congenic background. The remaining approximately 1.6 ml of extract was sonicated five time for 3 s, then 10^6 cpm of tracer [³⁵S]HS was added to correct for extraction losses. Glycosaminoglycans were cleaved from proteoglycans by addition of 36 µl of 5.6 M NaOH with 4.4 M sodium borohydride and refluxed at 46°C for 12 h. After centrifugation at 10,000 rpm for 20 min, ice-chilled supernatants were slowly added to 0.5 ml of 8.54 M ammonium formate containing 1.7 M HCl in a vortexed 15-ml tube. After centrifugation at 10,000 g, supernatants were extracted four times against 5 ml of phenol, three times against 7 ml of phenol/chloroform/isoamyl alcohol (25:24:1), and once each against 3 ml of chloroform/isoamyl alcohol (24:1) and 6 ml isobutanol. Glycosaminoglycans were precipitated with 5 ml ethanol, then harvested by centrifugation at 10,000 g for 1.5 h. Pellets were resuspended in 100 µl water, and chondroitin sulfate was degraded with 0.02 U of chondroitinase ABC (18). HS was purified by phenol extraction and ethanol precipitation (18), then mass was determined by forming complexes with Alcan blue (Fluka Chemical Corp., Milwaukee, Wisconsin, USA) (28) using kidney HS (ICN Biomedicals Inc., Costa Mesa, California, USA) as standard. Complexes were harvested by centrifugation at 10,000 g for 30 min, then resuspended in 100 µl of 8 M guanidine HCl with 0.1 % Triton X-100 and spectrophotometrically measured at A600.

**HSact conversion assay.** The HSact conversion assay (18) measures 3-OST-1 formation of AT-binding sites. In the presence of adenosine 3′-phosphate 5′-phosphosulfate (PAPS), 3-OST-1 converts [³⁵S]HS, lacking 3-O-sulfates, into [³⁵S]HSact, which contains AT-binding sites and is quantified by AT-affinity chromatography (9). [³⁵S]HS lacking 3-O-sulfates was prepared from metabolically labeled CHO-K1 cells, and reactions containing 80,000 cpm of [³⁵S]HS were assembled per Yabe et al. (25), with the following modifications to optimize sensitivity.
Plasma (1 µl) or lung homogenates (20 µg) were analyzed in reactions containing 0.4 mg/ml chondroitin sulfate C and lacking NaCl and glycogen. Reactions for brain (10 µg) or heart (40 µg) homogenates lacked chondroitin sulfate, NaCl, and glycogen. Activity was calibrated against purified recombinant 3-OST-1 standards, which were run in the absence and presence of plasma and tissue homogenates.

**Measurement of anti-Xa activity of tissue HS, plasma AT activity, and plasma T•AT complexes.** The in vitro activity of tissue HS to enhance AT neutralization of factor Xa was determined as described previously using S-2765 to monitor Xa activity (29). Activity was calibrated against a standard curve of porcine heparin (179 USP U/mg) (H-3393; Sigma-Aldrich). Plasma heparin cofactor II (HCII) activity was similarly detected using 205 nM human HCII with 125 nM human α-thrombin (Haematologic Technologies Inc., Essex Junction, Vermont, USA) and substrate S-2238 (Diapharma Group, West Chester, Ohio, USA). Plasma AT activity (anti-Xa activity) was measured with a Coamatic AT (Chromogenix Instrumentation Laboratory S.p.A., Milan, Italy) kit according to the manufacturer’s specifications and using purified AT (Cutter Laboratories, Berkeley, California, USA) as standard. Plasma T•AT level was measured with an enzyme immunoassay using the Enzygnost TAT micro kit (Dade Behring Inc., Deerfield, Illinois, USA).

**Tissue AT-binding activity.** Organs (10-mg portions) were extracted twice in 500 µl of 50 mM Tris-HCl buffer, pH 8.0, containing 8 M urea, 10 mM EDTA, 1 mM PMSF, and 1 M DTT, in a Potter homogenizer at room temperature. The pooled extracts were clarified by centrifugation for 30 min at 10,000 g, and the supernatants were filtered through a 0.22-µm Millex GV filter. The protein concentration of tissue extracts was determined using the bicinchoninic acid reagent (Pierce Chemical Co., Rockford, Illinois, USA). Aliquots of the tissue extracts containing 2–20 µg proteins were loaded in triplicate onto nitrocellulose membrane using a dot-blot apparatus, and 125I-AT ligand-binding assay was performed as described (30).

**In situ detection of AT-binding sites.** Tissue isolation, generation of cryosections, incubation of sections with 125I-labeled AT and autoradiography were all performed as described previously (31). Specificity of AT binding to HS was confirmed by competition with soluble sulfated polysaccharides and by preincubation with GAG lyases (31). Dark-field and phase-contrast images were recorded with a color CCD camera (SPOT model 1.3.0; Diagnostic Instruments, Sterling Heights, Michigan, USA) and manipulated with Adobe Photoshop 6.0 using identical exposure and manipulation settings for Hs3st1–/– and Hs3st1+/+ samples.

**Tissue fibrin levels.** Urea-insoluble tissue extracts (containing cross-linked fibrin) were prepared, and fibrin was measured by Western blot analysis with a fibrin-specific Ab, NYB T2G1, as described previously (32). Subsets of mice were subjected to overnight hypoxia (8% O2) to induce procoagulant changes in lung tissue, as described (33).

**Acute carotid artery injury.** FeCl3-induced arterial injury was performed similar to published procedures (34). Analyses of inbred mice lines showed that injury responses were very different for C57BL/6 versus 129S4/SvJae strains and that F2 hybrids yielded mice with extremely variable responses. Given this dependency on genetic background, studies were conducted with inipient congenic 129S4/SvJae mice only. Mice (25–35 g) were anesthetized with 1.25% Avertin (0.34 mg/g intraperitoneally), intubated, and ventilated (14 µl/g; 110 breaths/min). The right and left common carotid arteries were exposed by blunt dissection. Miniature Doppler flow probes (0.5VB; Transonic Systems Inc., Ithaca, New York, USA) were positioned around the distal limit of each common carotid artery and blood flow in both arteries was recorded simultaneously. Ten minutes after probe placement, the left carotid artery was chemically injured by applying a 1.0 × 0.6-mm strip of filter paper soaked in 30% FeCl3, to the proximal adventitial surface for 1 min. The field was flushed with saline, and flow was monitored until complete occlusion occurred. The injury procedure was then repeated on the right common carotid. Flow was measured with a Transonic T206 meter using a 30-Hz filter, and data were acquired with WinDaq software (DATAQ Instruments, Akron, Ohio, USA). Fast Fourier transformation identified the point at which flow was undetectable. Occlusion times were not correlated to initial blood flow rates (Hs3st1+/+ r2 < 0.001; Hs3st1–/– r2 = 0.0314), so data were not adjusted for initial flow. Injured arteries were collected in Bouin’s fixative, and platelet-rich thrombi were confirmed with hematoxylin and eosin staining of paraffin sections.

**3-OST-1 overexpression**

**Retroviral transduction.** CHO4B is a clonal line expressing the ecotropic retrovirus receptor (35). Primary mouse cardiac microvascular endothelial (CME) cells were described previously (20). pRV-3OST1 contains a 1.6-kb (Bgl II/Xho I) 3-OST-1 fragment from pCMV3-OST (20). pRV-AP contains a secreted alkaline phosphatase (AP) cDNA, a 1.7-kb BglII/HpaI fragment from pSEAP2-basic (CLONETECH Labs Inc., Palo Alto, California, USA). The cDNAs were inserted into pMIG (36) (courtesy of D. Baltimore, California Institute of Technology, Pasadena, California, USA) upstream of IRES-GFP. The resulting bicistronic message also produces green fluorescent protein. Ectropic retroviral vectors were generated with Phoenix vector and Phoenix cells as described previously (35). Both cell types were transduced by two successive exposures at a MOI of 0.75 (9), yielding approximately 90% of cells expressing green fluorescent protein.

**Analysis of transductants.** Previously, we described methods for measuring 3-OST-1 mRNA expression (20), 3-OST-1 enzyme activity (18), and HS–gly synthetic rate (9, 18). For cell surface activation of AT, multiwell
plates were inoculated at 50,000 cells per 1.9-cm² well. Assay background was determined with wells lacking cells. Two-day-old cultures were washed with PBS containing 5 mg/ml BSA and 40 nM human AT (Cutter Laboratories). Monolayers were incubated at room temperature for up to 1 h with 0.1 ml of PBS containing 40 nM AT and 5 nM human α-thrombin (Haematologic Technologies Inc.). At 10-min intervals, quadruplicate wells were evaluated for residual T activity by combining 65 µl of sample with 25 µl of 1.8 mM S-2238 (Diapharma Group) in PBS. After 10 min at room temperature, reactions were quenched with 65 µl glacial acetic acid, and A₄₀₅ was measured. Given the above pseudo–first-order conditions, exponential decay constants were determined and corrected for the initial AT concentration to provide apparent second-order rate constants (kₑ₂) for T neutralization (37).

Results

Generation of Hs3st1–/– mice. To examine whether hemostatic tone is tightly controlled by HS act levels, we generated Hs3st1–/– mice. Characterization of the mouse Hs3st1 gene revealed that the entire 3-OST-1 coding region was encompassed within exon 8. To generate mice deficient in 3-OST-1 enzyme, ES cells were electro-ported with a replacement vector that eliminates exon 8 (Figure 1a). Southern blot analyses of 534 G418-resistant ES cell clones with a 3′ probe revealed two homologous recombinants. Verification with a 5′ probe, however, demonstrated only a single clone had undergone appropriate 5′ recombination. Only this clone was devoid of exon 8, as revealed by a coding region probe. Further probing for the neomycin resistance gene showed that this clone was devoid of extraneous integrations. Injection of this clone into blastocysts generated chimeric mice that were germline competent. Chimeras were bred to C57BL/6J females, and interbreeding of heterozygotes resulted in viable mice (Figure 1b) with comparable recovery of nulls and the wild-types. Tissue homogenates and plasma were evaluated for 3-OST-1 activity by the HS act conversion assay, which measures formation of AT-binding sites within HS (Figure 1c). Consistent with the removal of the entire gene-coding region, enzymatic activity was virtually absent in all samples from Hs3st1–/– mice.

3-OST-1 is the predominate source of HS act. The effect of Hs3st1 disruption on in vivo levels of HS act was revealed by isolating HS from a variety of tissues. For each tissue examined, recovery of total HS was comparable between Hs3st1+/– and Hs3st1+/+ mice (data not shown). Indeed, given the scarcity of 3-O-sulfates within HS (9, 10), altered HS levels should not occur. For most Hs3st1–/– tissues examined, the in vitro anti-Xa activity of HS was reduced by 75–98%, as compared with Hs3st1–/+ material (Figure 2a). Probing blots of immobilized tissue extracts with 125I-AT revealed AT-binding sites in Hs3st1–/– mice were similarly reduced (data not shown). The extent of reduction closely correlated with tissue-expression levels of 3-OST-1 mRNA, which we have determined previously (22). The minor residual AT-binding sites and anti-Xa activity suggest other 3-OST isoforms can contribute to HS act production. For most tissues, however, 3-OST-1 is clearly the predominant isoform and produces the vast majority of AT-binding sites. Large reductions of HS act might modestly perturb plasma AT levels, given that 10–20% of total body AT is sequestered by vascular endothelial HS act (38). Removal of this compartment could produce a slight elevation in plasma AT. Consistent with a loss of vascular HS act, baseline plasma AT activity was marginally elevated, by approximately 15% (Hs3st1+/– 1.8 ± 0.05 U/ml, n = 12 versus Hs3st1+/+ 2.0 ± 0.12 U/ml, n = 7; P < 0.05).

Figure 1
Disruption of the mouse Hs3st1 locus. (a) Gene-targeting strategy. Exon 8 bridges two BamHI fragments and includes the entire coding sequence (white box) flanked by 5′ and 3′ noncoding sequences (dark gray boxes). The targeting construct replaces exon 8 with a neo expression cassette (light gray boxes) in the same transcriptional orientation. The disrupted locus lacks specific BamHI, SpeI, and BglII sites. Restriction sites for producing targeting arms are shown. Also indicated are the locations of 5′ and 3′ probes for Southern blot analysis genotyping and primer sites for PCR analysis genotyping. (b) Genotyping by Southern blotting. Mouse tail genomic DNA was digested with BamHI, then the Hs3st1 locus was detected with the external 5′ probe (shown in a). The wild-type allele generates a 5.6-kb band, whereas the disrupted allele generates a 12.7-kb band due to loss of a BamHI site. (c) 3-OST-1 activity of mouse plasma and tissue homogenates; n = 3 littermates per group. 3-OST-1 activity creates AT-binding sites within HS (producing HS act) and is determined by incubating tissue homogenate or plasma samples with [35S]HS and PAPS, then measuring the resultant [35S]HS act by AT-affinity chromatography.
Hs3st1–/– mice do not show an obvious procoagulant phenotype. Given the large reductions in HSact, Hs3st1–/– were expected to show a procoagulant phenotype. We measured the basal accumulation of fibrin within tissues, which is an extremely sensitive index of microvascular hemostatic balance (32, 33). Surprisingly, tissue fibrin levels for Hs3st1–/– mice were indistinguishable from Hs3st1+/+ littermates (Figure 2b). Wild-type fibrin accumulation even occurred in organs with extremely low residual levels of anti-Xa activity (e.g., Hs3st1–/– lung and kidney, which had reductions of ∼98%, Figure 2a). Thus, a basal procoagulant state was not detected.

In an attempt to uncover a latent procoagulant condition, mice were subjected to a procoagulant challenge — overnight hypoxia (8% O2). Prolonged hypoxia elevates expression of tissue factor in the monocyte/macrophage lineage and in pulmonary vascular endothelial cells, which leads to enhanced fibrin accumulation and pulmonary thrombosis (33, 39). Despite the large HSact reduction in Hs3st1–/– lung, hypoxia-induced fibrin accumulation was comparably elevated (∼2.5-fold) in lungs of control and 3-OST-1–deficient animals (Hs3st1+/+ 38.4 ± 4.0 µg/g tissue versus Hs3st1–/– 40.1 ± 4.4 µg/g tissue; n = 3 litters per group). Thus, a basal procoagulant state was not detected.

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occlusive thrombosis, so should allow detection of enhanced thrombosis (data not shown). Thrombi that formed in wild-type and knockout mice were comparable by gross histologic inspection (results not shown). Monitoring blood flow revealed that the time to generate a complete occlusion was indistinguishable between genotypes (Figure 3b, $P > 0.5$). A potential difference might be masked if lack of 3-O-sulfates enhances the HCII activity of HS. This possibility, however, was ruled out because the HCII activity of tissue HS was identical between genotypes (Figure 2a inset). Immediately after occlusion of both common carotid arteries, an intraventricular blood sample was drawn to measure T•AT complexes. The postinjury concentration of T•AT complexes within plasma was independent of mouse genotype (9.1 ± 1.2 µg/l versus 9.7 ± 1.0 µg/l for Hs3st1–/– and Hs3st1+/+, respectively; mice analyzed in Figure 3b). Thus, the profound reduction in subendothelial matrix HSact did not expedite occlusive thrombosis in Hs3st1–/– mice and did not alter postinjury levels of T•AT complexes in plasma.

3-OST-1 overexpression fails to enhance AT activation by the endothelial cell surface. As a complementary approach to assess the influence of HSact levels on coagulation, we overexpressed 3-OST-1 in primary mouse CME cells, which have endogenous 3-OST-1 expression (20), and in CHO4B, a nonendothelial cell line lacking 3-OST-1 (43). Cells were transduced with the retroviral vector pRV-3OST1 or the control vector pRV-AP, which respectively express CDNAs for 3-OST-1 or a secreted form of AP. The pRV-3-OST-1 transductants, compared with pRV-AP transductants, showed increased 3-OST-1 mRNA and enzyme levels, as revealed by Northern blotting and HS act conversion assays, respectively (data not shown). We then measured cellular synthesis of HS act and ability of cell monolayers to enhance AT neutralization of α-thrombin (Figure 4). For pRV-AP transductants, HS act production and α-thrombin inactivation were only evident in cells with endogenous 3-OST-1 expression (CME as opposed to CHO4B cells). pRV-3OST1 transduction elevated HS act synthesis in both cell types ($P < 0.001$, compared with pRV-AP transductants). Catalysis of AT activity, however, was only elevated for monolayers of CHO4B cells ($P = 0.001$), but not CME cells ($P > 0.2$). Although HS act can contribute to cell surface activation of AT in CHO4B, there does not appear to be a 1:1 correlation between HS act production and cell surface activity in CME cells. The lack of correlation might suggest that HS act is not the sole endothelial anticoagulant and/or that mechanisms exist to maintain a set anticoagulant tone on the endothelial surface. Regardless, these results are consistent with Hs3st1–/– data showing HS act levels and anticoagulant state are not tightly linked.

Hs3st1–/– mice have genetic background-dependent postnatal lethality. Hs3st1–/– mice are further remarkable because they develop several unanticipated abnormalities, including postnatal lethality and intrauterine growth retardation. Such phenotypes can arise from a gross coagulopathy (7, 44). Consequently, we examined whether perinatal traits of Hs3st1–/– mice stem from such a cause. These phenotypes were detected while altering strain genetic background. Knockout mice were initially generated on a mixed genetic background (C57BL/6 × 129S4/SvJae). The knockout allele was successively bred through C57BL/6 mice. After seven backcrosses, less than approximately 1% of the genome is derived from the original ES cell clone; thus, perinatal phenotypes are unlikely to have resulted from a secondary gene mutation. It remains conceivable that targeting of the Hs3st1 locus has perturbed the expression of adjacent genes; thus, we can not yet conclusively prove direct causation from loss of 3-OST-1 expression. The potential involvement of a gross procoagulant state, however, obligated our evaluation of these phenotypes.

Hs3st1–/– mice from various backcrosses were interbred to produce litters on progressively enriched C57BL/6

Figure 4
Overproduction of HS act in endothelial cells does not augment cell surface activation of AT. CME (Endothelial) and nonendothelial (CHO4B) cells were transduced with pRV-3OST1 (3OST1) or pRV-AP (AP) retroviral vectors. (a) HS act synthesis was determined by pulse-labeling cells 1 h with $[^35S]NaSO_4$ followed by HS purification and AT-affinity chromatography ($n = 3$ independent labelings). Incorporation rate into HS act is expressed as AT-bound $[^35S]$HS, standardized to cellular protein. (b) Cell surface catalysis of AT activity was determined by incubating monolayers with α-thrombin and excess AT. At various times, residual α-thrombin activity was chromogenically determined and apparent second-order rate constants ($k_r^*$) for T inactivation were derived ($n = 4$ independent experiments). Monolayer-dependent catalysis was determined by subtracting the assay background of 3,800 M–1 s–1 (obtained by incubations without monolayers). Comparable data were also obtained for AT neutralization of factor Xa (not shown).
 backgrounds. Surprisingly, the recovery of Hs3st1/−− weanlings dramatically diminished as C57BL/6 content increased (a representative lineage presented in Table 1). After four backcrosses, recovery of Hs3st1/−− weanlings bottomed out at approximately 10% of Hs3st1/+ levels (Table 1; compare N4 to N7). Hs3st1/−− mice showed a partial effect with recovery being approximately 35% of expected (N4 + N7 litters had 30 Hs3st1/−−, so should have produced 60 Hs3st1/−−). Hs3st1/−− lethality persisted even when litters were produced by embryo transfer into the high-fecundity mouse strain FVB/N [Table 1; N7 (ET)]. Thus, lethality was predominantly dependent on offspring genotype rather than maternal genotype. The requirement for an enriched C57BL/6 background was further confirmed by breeding Hs3st1/−− mice from the eighth generation backcross (N8) to Hs3st1/−− mice produced on an incipient congenic 129S4/SvJae background. Hs3st1/−− lethality was completely rescued by the resulting hybrid background.

AT-deficient (ATIII−/−) mice have intrauterine lethality with no survival past E16.5 (7). Given that a gross hypercoagulable state is causative, we examined if Hs3st1/−− lethality mimics AT deficiency; however, Hs3st1/−− mice showed normal viability 1 day before birth (E18.5) with only slight reductions within 48 h of delivery (Table 1; genotype analysis of P0/P1 at N6). The lethality is probably complete by postnatal day 2–3 (P2–P3) because reductions in litter size were frequently observed during this period (results not shown). Thus, in contrast to ATIII−/− mice, Hs3st1/−− mice exhibited postnatal rather than intrauterine lethality. In humans, AT deficiency and other hypercoagulable states can lead to postnatal lethality from purpura fulminans (6); however, bruising and subcutaneous hemorrhages were not evident in newborn Hs3st1/−− pups, despite being subjected to normal birth trauma. Furthermore, Hs3st1/−− newborns had unlabored breathing, ingested milk, produced and excreted urine, and exhibited normal startle reflexes to noise and motion. Thus, a gross cause for postnatal lethality was not readily apparent. Histological surveys of E18.5 and P0 mice failed to reveal thrombosis, hemorrhage, or anatomical anomalies in Hs3st1/−− embryos. Most importantly, myocardial and hepatic tissues lacked focal thrombosis and degeneration (data not shown), which invariably occurs in late-stage ATIII−/− embryos (7). Thus, Hs3st1/−− lethality is distinct from ATIII−/− lethality.

Hs3st1/−− embryos show intrauterine growth retardation. Although anatomical malformations were not apparent, Hs3st1 genotype did influence embryonic mass in a dose-dependent fashion. Compared with wild-type mice, Hs3st1+/− and Hs3st1−/− E18.5 embryos were 8% and 20% underweight, respectively (Figure 5a), and thus exhibited intrauterine growth retardation (IUGR). Embryo/placental-disk ratios, however, which are typically elevated in placental insufficiency, were comparable between genotypes (Hs3st1+/− and Hs3st1−/− ratios were 19.7 ± 0.8 versus 20.8 ± 1.1, respectively). IUGR from placental insufficiency also usually shows sparing of head growth (asymmetric IUGR) (46, 47). Yet Hs3st1−/− embryos, compared with Hs3st1+/− gestation mates, showed a significant reduction in the biparietal diameter (Figure 5b) — an established parameter of embryonic head growth (48).

Table 1

<table>
<thead>
<tr>
<th>Parental background</th>
<th>Offspring C57BL/6 content</th>
<th>Offspring Hs3st1 genotype</th>
<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
<th>+/+:−/− ratio</th>
<th>Loss of Hs3st1−/−</th>
<th>P&lt;sup&gt;C&lt;/sup&gt;</th>
<th>Age&lt;sup&gt;D&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>−50.0%</td>
<td>35</td>
<td>106</td>
<td>35</td>
<td>1:1</td>
<td>1.00</td>
<td>&lt;0.001</td>
<td>E18.5</td>
<td>P21</td>
</tr>
<tr>
<td>N3</td>
<td>−87.5%</td>
<td>21</td>
<td>45</td>
<td>5</td>
<td>1:2.40</td>
<td>76%</td>
<td>&lt;0.003</td>
<td>P21</td>
<td></td>
</tr>
<tr>
<td>N4</td>
<td>−93.8%</td>
<td>16</td>
<td>13</td>
<td>1</td>
<td>1:0.96</td>
<td>94%</td>
<td>&lt;0.001</td>
<td>P21</td>
<td></td>
</tr>
<tr>
<td>N6</td>
<td>−98.4%</td>
<td>10</td>
<td>17</td>
<td>12</td>
<td>1:1</td>
<td>1.00</td>
<td>E18.5</td>
<td>P0 + P1</td>
<td></td>
</tr>
<tr>
<td>N6 (ET)</td>
<td>−98.4%</td>
<td>18</td>
<td>26</td>
<td>11</td>
<td>1:1.60</td>
<td>39%</td>
<td></td>
<td>P14</td>
<td></td>
</tr>
<tr>
<td>N7 (ET)</td>
<td>−99.2%</td>
<td>14</td>
<td>8</td>
<td>2</td>
<td>1:1.04</td>
<td>86%</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N8&lt;sup&gt;mix&lt;/sup&gt; × 129S4/SvJae female</td>
<td>50.0%</td>
<td>25</td>
<td>56</td>
<td>22</td>
<td>1:1</td>
<td>1.00</td>
<td></td>
<td>P21</td>
<td></td>
</tr>
</tbody>
</table>

*N indicates number of successive backcrosses to generate parental Hs3st1+/+; ET indicates litters were generated by embryo transfer into FVB/N females. *Loss expressed relative to Hs3st1+/+. *Probability of a non-Mendelian outcome determined by χ<sup>2</sup> test. *Age at which tissue was collected for genotyping. P, postnatal day; E, embryonic days after conception.

Figure 5

Hs3st1+/− embryos exhibit IUGR. Hs3st1+/− mice from the N6 backcross were interbred, and the resulting embryos harvested at E18.5 were fixed, as described in Methods. Embryo mass without placenta (a) and head diameter (b) were then measured. *P < 0.05 and **P < 0.001, compared with Hs3st1+/+ gestation mates. n = 10 Hs3st1+/−, 17 Hs3st1−/−, and 12 Hs3st1−/− embryos.

Downloaded from http://www.jci.org on January 6, 2018. https://doi.org/10.1172/JCI15809
To assess whether reductions in head growth and embryonic mass were proportionate, we calculated a modified ponderal index (embryo mass + [biparietal diameter]^3). This index was indistinguishable between genotypes (Hs3st1^+/+, Hs3st1^-/-, and Hs3st1^-/- values were 3.9 ± 0.20, 4.0 ± 0.19, and 4.1 ± 0.18 g/mm^3, respectively) indicating that Hs3st1^-/- mice exhibit symmetric IUGR. Thus, the IUGR of Hs3st1^-/- embryos was not suggestive of placental vascular insufficiency. In a mouse model of thrombotic placental insufficiency that produces a comparable degree of IUGR (E18 embryos being 20% underweight), placenta exhibit fibrin thrombi and congestion (49). However, Hs3st1^-/- placentae were microscopically normal (data not shown). Nor was there evidence of giant cell hyperplasia, a feature of severe placental ischemia. Taken together, the data suggest the IUGR of Hs3st1^-/- mice did not stem from an overt procoagulant state. The contribution of IUGR toward neonatal lethality of Hs3st1^-/- mice warrants further investigation.

**Discussion**

To examine if hemostatic tone is tightly controlled by HS^act^ levels, we generated Hs3st1^-/- mice. The lack of 3-OST-1 enzyme causes large reductions in HS^act^ levels in most organs. Hs3st1^-/- mice were evaluated by techniques capable of detecting altered hemostatic balance (7, 32–34, 40, 41). Despite removing the majority of HS^act^, basal fibrin levels were completely unaltered in 3-OST-1–deficient mice. Even after a strong procoagulant stimulus (hypoxic challenge), lung fibrin levels in knockout mice were indistinguishable from wild-type mice. These results are particularly surprising given the dynamic nature of hemostatic balance. However, most of HS^act^ occurs in the subendothelial matrix. Consequently, an anticoagulant role might only be operable in the context of endothelial denudation, which would allow direct contact of the blood and matrix. But evaluation of Hs3st1^-/- and Hs3st1^-/- mice by an arterial injury model revealed both genotypes to have comparable vessel occlusion times and equivalent postinjury levels of T•AT complexes. Taken together, these analyses indicate that 3-OST-1–deficient mice lack an obvious procoagulant phenotype. Thus, hemostatic balance was not dependent on HS^act^ levels.

As a complementary approach, we employed 3-OST-1 overexpression to examine the effect of HS^act^ production on cell surface catalysis of AT activity. Overexpression conveyed both properties to cells lacking endogenous 3-OST-1. In endothelial cells, however, 3-OST-1 overexpression failed to enhance the preexisting high-catalytic activity of the cell surface, despite augmenting HS^act^ synthesis threefold. Again, anticoagulant activity was not tightly linked to HS^act^ production levels. This lack of correlation is consistent with observations that HS^act^ is not the sole endothelial component capable of catalyzing AT activity (12, 13).

Failure to detect a procoagulant phenotype in Hs3st1^-/- mice indicates HS^act^ is not a major hemostatic regulator like AT, thrombomodulin, or fibrinolytics. With these potent regulators, even a heterozygotic deficiency leads to fibrin elevations/hypercoagulable state (3, 5, 6). Our data, however, do not rule out some anticoagulant role for HS^act^. First, 3-OST-1–deficient mice maintain a small residual amount of HS^act^.

Residual HS^act^ is likely produced by other members of the 3-OST multigene family. For example, the liver has high residual HS^act^ and high expression of 3-OST-3a and 3-OST-3b. These isoforms, however, show low efficiency when generating HS^act^ (22, 25). Conversely, we have recently identified the final gene family members, 3-OST-5 and 3-OST-6. The latter is most homologous to 3-OST-1 in both genomic organization and protein structure. Moreover, 3-OST-6 is nearly as efficient as 3-OST-1 in generating HS^act^ (Rhodes, HajMohammadi, Seeberger, McNeely, Spear, and Shworny, unpublished data). 3-OST-6 might play an important role if hemostatic balance requires only low levels of HS^act^.

For example, HS^act^ on the endothelial luminal surface is present in relatively minute amounts (14, 15), but such localization might be essential for anticoagulant activity. Due to assay-sensitivity limitations, it is presently unclear whether 3-OST-1 deficiency affects luminal HS^act^.

Second, anticoagulant/fibrinolytic knockout mice show enhanced fibrin accumulation in a distinct spectrum of tissues (26, 32–34). The tissues examined in our present study have been sufficient to demonstrate a procoagulant state in such knockout mice. But potentially 3-OST-1–generated HS^act^ might serve an anticoagulant role in only a minor tissue not yet examined. Third, compensatory mechanisms might have masked a hemodynamic perturbation. Unmasking of an overt procoagulant effect might require an additional genetic defect. For example, combining the deficiencies for thrombomodulin and tissue plasminogen activator leads to an extreme hypercoagulable state with myocardial necrosis and depressed cardiac function (32). We are presently evaluating the above possibilities.

Our results do not exclude HS^act^ as a natural anticoagulant but instead suggest that the majority of HS^act^ is not essential for normal hemostatic tone. If the murine situation reflects human physiology, then the large number of hypercoagulable patients with undefined etiology (3, 4) cannot be the result of a deficiency in just 3-OST-1. It remains conceivable, however, that human 3-OST-1 deficiency might exert an affect when combined with deficiencies for other 3-OST isozymes or other anticoagulants/fibrinolytics.

Instead of an anticipated procoagulant state, Hs3st1^-/- mice exhibited unexpected phenotypes. Here we characterized two examples that do not appear to be of gross procoagulant origin. First, the Hs3st1^-/- mice showed genetic background-dependent lethality that is very distinct from the intrauterine lethality observed in ATIII^-/- mice. Hs3st1^-/- lethality occurred postnatally and was rescued on the mixed genetic background permissive for ATIII^-/- lethality. Moreover,
Hs3st1−/− embryos lack the gross and microscopic features of consumptive coagulopathy found in ATIII−/− embryos. Second, Hs3st1−/− embryos had IUGR. IUGR has been associated with placental insufficiency induced by procoagulant states, albeit typically of maternal origin (45, 50). Hs3st1−/− embryos, however, did not exhibit features of placental vascular insufficiency. Moreover, Hs3st1−/− placenta lacked microscopic evidence of thrombotic vascular insufficiency. Lethality might be secondary to IUGR, because lactating mice can preferentially kill low–birth weight mice. The mechanism by which lactation increases lethality is not known. The above data show that the above mice are consistent with the above method of litter “quality control.” Regardless, both lethality and IUGR of Hs3st1−/− mice did not appear to result from a gross procoagulant state. Potentially, these phenotypes might reflect a novel activity of HSact or of other 3-OST-1–generated structures. Evaluation of this possibility will require a comprehensive characterization of embryonic expression sites, because we have determined that 3-OST-1 transcripts are expressed throughout all stages of embryonic development (HajMohammadi and Shworak, unpublished data). Moreover, complementation studies must be conducted to confirm that these phenotypes are directly dependent on 3-OST-1 deficiency.

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