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Heparan Sulfate Ndst1 Regulates Vascular Smooth Muscle Cell Proliferation, Vessel Size and Vascular Remodeling

Neeta Adhikari, PhD^{1,2}, David L. Basi, DMD, PhD⁶, DeWayne Townsend, DVM, PhD^{1,7}, Melissa Rusch, PhD^{3,4,5}, Ami Mariash, MS^{1,2}, Sureni Mullegama, BS^{1,2}, Adrienne Watson, BS^{3,4,5}, Jon Larson, BS⁴, Sara Tan, BS^{1,2}, Ben Lerman, BS^{1,2}, Jeffrey D. Esko, PhD⁷, Scott B. Selleck, MD^{3,4,5}, and Jennifer L. Hall, PhD^{1,2,3}

¹ Lillehei Heart Institute, University of Minnesota, Minneapolis

² Department of Medicine, University of Minnesota, Minneapolis

³ Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

⁴ The Developmental Biology Center, University of Minnesota, Minneapolis, MN

⁵ Department of Pediatrics, University of Minnesota, MN

⁶ Department of Developmental/Surgical Science, University of Minnesota, Minneapolis, MN

⁷ Department of Integrative Biology and Physiology, University of California San Diego, La Jolla, California

⁸ Department of Cellular and Molecular Medicine, University of California San Diego, La Jolla, California

INTRODUCTION

Heparan sulfate proteoglycans are abundant molecules in the extracellular matrix and cell surface, consisting of a proteoglycan core protein attached to heparan sulfate chains¹. Heparan sulfate chains are composed of alternating N-acetylglucosamine and glucuronic acid moieties. The fine structure of the heparan sulfate chains are modified through a series of enzymatic reactions¹. Heparan sulfate serves as a docking site for multiple chemokines, lipids, and growth factors^{2,3}. We hypothesized that the fine structure of the heparan sulfate chain influenced proliferative properties of the VSMC. This hypothesis was based on previous findings in transgenic models in which heparan sulfate modifying enzymes had been deleted resulting in altered proliferation of different cell types^{4–11}.

Expression of one of the many heparan sulfate modifying enzymes, Ndst1, is up-regulated 40 fold in response to vascular injury¹². Ndst1 catalyzes an initial step in heparan sulfate modification - replacing N-acetyl groups with sulfate on the N-acetylglucosamine residues^{1,13}. We utilized two cre recombinase mouse models to delete Ndst1 in smooth muscle to test the role of Ndst1 in vascular remodeling. The two promoters used to express cre recombinase

Corresponding Author: Jennifer L. Hall, Ph.D. Lillehei Heart Institute, 4-106 NHH, 312 Church St SE, Minneapolis, MN 55455, Voice: 612.626.4566, Fax: 612.624.8118, jlhall@umn.edu.

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in smooth muscle were SM22 α and SMMHC. The SM22 α -cre⁺Ndst1^{-/-} mouse exhibited efficient and specific loss of Ndst1 in smooth muscle. As previously described in the literature¹⁴, the SMMHC-cre⁺Ndst1^{-/-} mouse exhibited ectopic recombination of floxed alleles in the germline and less efficient expression of cre-recombinase in smooth muscle compared to the SM22 α promoter. Although loss of a single allele in the germline and less efficient deletion in smooth muscle in the SMMHC-cre⁺Ndst1^{-/-} was not a fatal flaw, this model was limited to initial studies. Compromised Ndst1 expression led to significantly reduced lesion formation in response to injury in both models. Additional studies in the targeted SM22 α -cre⁺Ndst1^{-/-} lines showed a 50% loss of VSMC and decreased size of the femoral artery that was evident at day 7 which persisted through till adulthood. These findings may have important implications for the role of heparan sulfate in vascular structure and remodeling.

MATERIALS AND METHODS

Generation of Ndst1 deficient mouse models

Ndst1 deficient mice Ndst1^{flox/flox} mice were mated with male SM22 α -cre mice (gift from Dr. M. Parmacek). F1 cre⁺Ndst1^{wt/flox} males were mated with Ndst1^{flox/flox} females to generate mice with smooth muscle specific deletion of Ndst1. Ndst1^{flox/flox} mice were also backcrossed to a line of transgenic mice expressing Cre recombinase under the control of the smooth muscle heavy chain promoter (SMMHC-cre)¹⁵ (gift from Dr. M. Kotlikoff). This mating resulted in whole body deletion of one floxed allele of Ndst1 and loss of Ndst1 in smooth muscle. The phenotype resulting from mating with this line of SMMHC-cre mice has been characterized^{14, 16}. Genotype of the control mice used for the study was cre⁻ Ndst1^{+/+}.

The following primers were used to genotype the Ndst1^{flox}, Ndst1^{wt} and Ndst1^{-/-} alleles: Ndst1 (1–10R) 5'-ccagggcgtcagggcctctg-3', Ndst1 (1–16R) 5'-catcctctgaggtgaccgc-3', Ndst1 (1–17F) 5'-tcccatatggcgactgaggttc-3'⁸. Ndst1^{+/+} and Ndst1^{flox} alleles were identified by primer pair 1–10R and 1–17F while Ndst1^{-/-} allele was genotyped using 1–16R and 1–17F. Mice harboring deleted Ndst1 allele were also genotyped for Cre using primers: 5'-ccaatttactgaccgtacacc-3'; 5'-gtttcactatccaggttacgg-3'}¹⁵.

HPLC analysis of heparan sulfate disaccharide

Aorta were harvested from 16–20 weeks old males from all the cohorts and total heparan sulfate were isolated and profiled by HPLC according to the protocol described in Toyoda et al¹⁷. In brief, heparan sulfate isolated from each sample was enzymatically digested with a heparan lyase mixture into disaccharides. The disaccharides were then separated by reverse-phase ion-pairing HPLC, which allows for the quantification of six distinct disaccharide species. These include one unsulfated disaccharide, $\Delta^{4,5}$ -uronic acid-N-acetylglucosamine (D0A0), two monosulfated disaccharides: $\Delta^{4,5}$ -uronic acid-N-sulfated glucosamine (D0S0) and $\Delta^{4,5}$ -uronic acid-N-acetylglucosamine-6-O-sulfate (D0A6), two disulfated disaccharides: $\Delta^{4,5}$ -uronic acid-N-sulfoglucosamine-6-O-sulfate (D0S6), and 2-O sulfated $\Delta^{4,5}$ -uronic acid-N-sulfoglucosamine (D2S0), and one trisulfated disaccharide: 2-O sulfated $\Delta^{4,5}$ -uronic acid-N-sulfoglucosamine-6-O-sulfate (D2S6). The classification of unsaturated disaccharides is described in Lawrence et al¹⁸.

Assessment of Hemodynamic and Vascular Function

Mice were induced and maintained using isoflurane. Mice (n=4 in each cohort) were ventilated and the chest was opened to expose the heart. Following the removal of the pericardium and volume calibration procedures, a 1.2 Fr PV catheter (Scisense) inserted into the left ventricle via a stab incision in the apex of the heart. Following the surgical interventions, isoflurane was reduced to 1% for the functional measurements of baseline hemodynamics.

Surgical Intervention to induce Vascular Lesion

Animals were anaesthetized and all surgical procedures were in accordance with institutional IACUC guidelines and best practices for small animal surgery. A straight guide wire (0.38 mm in diameter) was inserted into the left femoral artery of 16–20 weeks old male mice via a small muscular branch as previously described^{19–21}. The wire was left in the lumen for 1 minute to denude and dilate the artery. After removing the wire the small branch was tied off and blood flow was restored in the injured vessel.

Morphometry

Mice were perfused with PBS followed by 10% neutral-buffered Formalin using the, “In Vivo Rodent Perfusion System”(Automate Scientific). Morphometric evaluation along the length of femoral arteries harvested at 0 and 28 days post injury was performed by staining 5 μ m paraffin embedded sections with hematoxylin-eosin (H&E) and examination under an Olympus BX41 microscope. Equally spaced sections (8–10 per mouse), covering 500 μ m of femoral artery length were measured for Internal Elastic Lamina (IEL), External Elastic Lamina (EEL) lengths, for lumen, for intimal and medial areas. Sections of femoral arteries from all the cohorts were also stained with Verhoeff-Van Gieson stain for better visualization of IEL and EEL. All the measurements were done using the NIH ImageJ software. Morphometric analyses of newborn pups were done as mentioned above.

Immunohistochemistry

Immunohistochemical localization of N-sulfated epitopes on native HS chains was performed on 5 μ m sections of injured and non-injured femoral arteries. Three randomly chosen sections per animal were stained for N-sulfated epitopes by using 5 μ g/mL of anti-mouse HS-10E4 (Seikagaku, Japan) as previously described²². Anti-mouse HS-10E4 specifically recognizes an epitope containing an N-sulfated residue in native HS chains. Staining intensity in femoral artery section was measured by using Photoshop 7.0. The intensity was plotted as a ratio of brown stained area pixels to total intimal area pixels. For quantification of collagen content 5 μ m sections of femoral arteries 28 days post injury from all the cohorts were stained for Masson's Trichrome staining. Collagen content was quantified by using Photoshop 7.0 and plotted as a ratio of blue stained pixels to total intimal area pixels. VSMCs were counted following staining with anti Smooth Muscle α -actin (Sigma).

Western Analysis

Aortas from control, SMMHC-cre⁺Ndst1^{-/-} and SM22 α -cre⁺Ndst1^{-/-} mice were lysed in RIPA buffer containing protease inhibitors. Tissue or VSMC lysates were run on a 10% gel and Ndst1 protein was detected with anti-Ndst1 N20 antibody (Santa Cruz Biotechnology). Anti-mouse β -actin (Sigma-Aldrich) was used a loading control.

Cell Proliferation

Cell proliferation 3 days post injury in femoral arteries was assessed by Bromodeoxyuridine (BrdU) incorporation according to the manufacturer's instructions (Roche). Control and SM22 α -cre⁺Ndst1^{-/-} mice were injured as previously described^{19–21} and harvested three days post injury. One hour prior to sacrifice, mice were injected with BrdU (ip, 1–2mL/100 g body weight). Sections were processed according to the manufacturer's instructions. Proliferating VSMC in neonatal femoral arteries was assessed by staining with Ki67 (Thermo Scientific). Cells were visualized following secondary staining with anti rabbit Alexa fluor 594 (Invitrogen). Proliferating VSMCs *in vitro* were assessed with [3H]-Thymidine as previously described²³. Briefly, A7r5 VSMC were treated with 75 mM sodium chlorate or vehicle in the presence of 10% serum for 24 hours, [3H]-Thymidine (1.0 mCi/ml) was added for the last four hours.

Real Time quantitative PCR

RNA was extracted from tissues and VSMCs using the RNeasy kit (Qiagen) and reverse transcribed to cDNA (Advantage RT-PCR, BD Biosciences). Real Time quantitative PCR utilizing Applied Biosystems primer probe protocol was performed with the following targets: *Ndst1* (Mm01262753_m1), *Ndst2* (Mm00447818_m1), *Ndst3* (Mm00453178_m1), *Ndst4* (Mm00480767_m1), *HPRT1* (Mm01545399_m1). All target gene expressions were normalized to *HPRT1*, an internal control. *HPRT* expression was unchanged between models. Analysis was performed in the ABI7900. Gene expression was calculated using the delta delta Ct method as previously reported ²⁴.

Isolation and culturing of aortic VSMCs was done according to the protocol described in Ray et al ²⁵.

Statistical Analysis

All values are represented as mean $\pm$ SE. Statistical significance compared to *cre⁻Ndst1^{+/+}* control values was analyzed by one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The goals of this study were to determine the role of heparan sulfate modification on VSMC proliferation, vessel size and vascular remodeling in response to injury in neonatal and adult mice. To do this we utilized two genetic models with deleted expression of *Ndst1*.

The two murine models are described in the Materials and Methods section. Briefly, the *SM22 α -cre⁺Ndst1^{-/-}* mouse exhibits targeted loss of *Ndst1* in smooth muscle. The *SMMHC-cre⁺Ndst1^{-/-}* mouse exhibits a single allele of *Ndst1* throughout all tissues and decreased expression of *Ndst1* in smooth muscle. Transcript abundance of *Ndst1* was decreased in femoral arteries of the *SMMHC-cre⁺Ndst1^{-/-}* and *SM22 α -cre⁺Ndst1^{-/-}* mice (Figure 1a). *Ndst1* transcript levels in isolated VSMCs was decreased in *SMMHC-cre⁺Ndst1^{-/-}* and *SM22 α -cre⁺Ndst1^{-/-}* compared to *cre⁻Ndst1^{+/+}* mice (Online Figure 1A). *Ndst1* protein in *SM22 α -cre⁺Ndst1^{-/-}* aorta and in isolated VSMCs from *SMMHC-cre⁺Ndst1^{-/-}* was also decreased compared to controls (Figure 1b). Disaccharide profiles performed with HPLC provide a functional measurement of loss of function of *Ndst1* in mouse aorta. The *SM22 α -cre⁺Ndst1^{-/-}* mice exhibited the most significant loss of N- and 2-O sulfation (3.6 fold and 4.5 fold, respectively). Comparatively, the *SMMHC-cre⁺Ndst1^{-/-}* mice exhibited a 2 fold decrease in N- and a 1.5 fold decrease in 2-O sulfation (Figure 1c). A cartoon diagram of a native heparan sulfate chain and a heparan sulfate chain generated in the absence of *Ndst1* is shown for comparison in Figure 1d. Note the loss of N-sulfated residues spaced throughout the length of the chain. Immunostaining for N-sulfated epitopes provides a second means for detecting loss of *Ndst1* in the vessel. Using the well-characterized antibody HS-10E4 ²², which stains N-sulfated epitopes, we confirmed the significant loss of N-sulfated heparan sulfate epitopes in vessels from both models (Online Figure 1B-D). Quantification of the staining intensities is shown in Online Figure 1E. Note, the transcript abundance of *Ndst2* did not change with loss of *Ndst1* (control 0.30 ± 0.04 (n=7); *SMMHC-cre⁺Ndst1^{-/-}* 0.37 ± 0.07 (n=3); *SM22 α -cre⁺Ndst1^{-/-}* 0.26 ± 0.03 (n=3)). *Ndst3* and 4 expressions were undetectable, as we have previously reported ¹².

To assess the consequence of the loss of smooth muscle *Ndst1* on the functional characteristics of the heart hemodynamic measurements were performed in *SM22 α -cre⁺Ndst1^{-/-}* mice. Systolic pressure and Left Ventricular End-diastolic Pressure (LVEDP) were not significantly different in *SM22 α -cre⁺Ndst1^{-/-}* mice (Figure 2a and b). In addition, ejection fraction (control

37 ± 3.5 ; SM22 α -cre⁺Ndst1^{-/-} 46 ± 8 , $p=ns$) was not altered at baseline in SM22 α -cre⁺Ndst1^{-/-} ($n=4$) compared to age matched controls ($n=4$).

Histomorphological evaluation of femoral arteries from neonatal and adult SM22 α -cre⁺Ndst1^{-/-} mice revealed decreased lumen area of the vessels, and reduced length of both the IEL and EEL as compared to controls (Table 1). The number of VSMCs in the neonatal and adult femoral arteries showed a ~ 50% decrease in SM22 α -cre⁺Ndst1^{-/-} mice (Figure 2c). Proliferating VSMCs were significantly reduced in SM22 α -cre⁺Ndst1^{-/-} neonatal femoral arteries compared to control femoral arteries (Figure 2d). Proliferating VSMCs were not detected in femoral arteries of adult mice. No change in VSMC number or vessel size was seen in the adult SMMHC-cre⁺Ndst1^{-/-} mice (data not shown). This coincided with the increased expression of Ndst1 mRNA and N- and 2-O-sulfated disaccharides compared to the SM22 α -cre⁺Ndst1^{-/-} mice as shown in Figures 1a and b.

To study the effect of Ndst1 deletion in vascular remodeling, a well-characterized wire injury was performed^{19–21}. At 28 days post injury, the intima/media ratio was decreased in both SM22 α -cre⁺Ndst1^{-/-} and SMMHC-cre⁺Ndst1^{-/-} mice compared to control mice (Figure 3a–d). The intimal area in both the models was significantly reduced in response to injury. Detailed morphometry measurements in both the models are listed in Table 2. To test the hypothesis that loss of N-sulfated disaccharides inhibited VSMC proliferation in response to injury, BrdU incorporation assays were performed. Three days post injury, BrdU incorporation in medial VSMCs was decreased by 50% in SM22 α -cre⁺Ndst1^{-/-} mice compared to control mice (Figure 3e). In parallel, in vitro inhibition of sulfation decreased proliferation in rat embryonic smooth muscle cells (Figure 3f).

Quantification of the staining intensity of N-sulfated epitopes was significantly reduced in the lesion from the SM22 α -cre⁺Ndst1^{-/-} as compared to control mice (Online Figure II). In addition, collagen was also decreased in both the models compared to control mice (Figure 4a–c). Quantification of the loss of collagen content in the lesion in SMMHC-cre⁺Ndst1^{-/-} and SM22 α -cre⁺Ndst1^{-/-} mice is depicted in Figure 4d.

DISCUSSION

We utilized two genetic mouse models; SMMHC-cre⁺Ndst1^{-/-} and SM22 α -cre⁺Ndst1^{-/-} to study the role of Ndst1 in vascular remodeling. Both the models exhibited significant reduction in N- and 2-O-sulfated disaccharide species along the heparan sulfate chain. In response to injury both the models showed significantly smaller lesions in association with reduced collagen content. Vessels of SM22 α -cre⁺Ndst1^{-/-} which showed a greater loss of N- and 2-O-sulfated disaccharides exhibited a ~ 50% reduction in VSMC number and proliferation. In this study we provide new evidence that the level of N- and 2-O sulfation of heparan sulfate is critical in the regulation of VSMC proliferation and determination of vessel size.

Heparan sulfate serves as a docking site for multiple chemokines, lipids, and growth factors^{2, 3}, yet its function is often overlooked. The pathobiology of diseases including atherosclerosis and diabetes has been associated with alterations in heparan sulfate proteoglycans^{26–28}. The fine structure of heparan sulfate is modified by a number of enzymatic reactions^{1, 29–31}.

How the fine structure of heparan sulfate influences tissue structure and risk of disease is a newly recognized area in biology. New data presented in this study suggests that N- and 2-O-sulfated disaccharides contribute to VSMC proliferation, vessel size and vascular remodeling in response to injury. Heparan sulfate proteoglycans have been shown to serve as co-receptors for Wnt ligands³. Earlier work from our laboratory demonstrated a role for β -catenin and Tcf-4 (Tcf7l2) in vascular remodeling²³. The Wnts involved in vascular remodeling have remained

elusive. Ongoing studies in our laboratory are focused on the role of Wnt/Heparan sulfate signaling in vascular development and remodeling.

Systemic deletion of *Ndst1* results in perinatal lethality characterized by severe developmental defects of the forebrain, eyes and lung ^{4, 5, 8}. Disaccharide profiling of *Ndst1* knockouts have demonstrated a similar decrease in N- and 2-O sulfated disaccharides ³². In the present study we utilize a sensitive HPLC technique to separate the specific disaccharide profile and show a ~ 4-fold decrease in N- and 2-O sulfated disaccharides in the SM22 α -cre⁺*Ndst1*^{-/-} mice. This specific disaccharide profile was associated with a reduction in proliferating VSMC and decreased vessel size in neonatal and adult mice. The SMMHC-cre⁺*Ndst1*^{-/-} mouse showed a ~ 1.5-fold decrease in N- and 2-O sulfated disaccharides. These mice did not show a decrease in VSMC number or vessel size. In this study we provide new evidence that N- and 2-O sulfation of heparan sulfate is critical in the regulation of VSMC proliferation and determination of vessel size. These findings confirm previously published work in other tissues/organs showing decreased cell proliferation in brain, endothelium, and bone of mice lacking *Ndst1* ^{33, 34}.

Early studies demonstrated that purified and exogenous administration of heparin, a highly sulfated heparan sulfate; inhibit migration and proliferation of VSMC in response to vascular injury ^{35–39}. In addition, PI-88 a heparan sulfate mimetic also suppressed VSMC proliferation in response to injury by binding FGF2 and blocking cellular signaling ⁷. Tran et al demonstrated increased VSMC proliferation in transgenic mice harboring a heparan sulfate deficient perlecan in response to injury ⁴⁰. Baker et al. recently reported that overexpression of heparanase which cleaves heparan sulfate chains resulted in increased medial thickness and an increased lesion in response to injury ⁴¹. The data presented in this study provide evidence that modification of heparan sulfate fine structure, such that N and 2-O sulfation is significantly reduced in VSMC, results in a significant decrease in proliferating VSMCs and decrease in vessel size in neonatal mice. Taken together, these findings all suggest that heparan sulfate chains regulate VSMC proliferation. This is the first study to identify early changes in VSMC proliferation and vessel size as a result of specific modifications in heparan sulfate chains.

In summary, this study adds a new perspective to the current view of the role of heparan sulfate fine structure in vascular biology. In this study, we show that modification of the disaccharides on the heparan sulfate chains (loss of N- and 2-O sulfation) is sufficient to decrease VSMC proliferation, vessel size and the response to remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of Ndst1 deficient mouse models. (a) Ndst1 transcript abundance in adult femoral arteries of control, SMMHC-cre⁺Ndst1^{-/-} and SM22α-cre⁺Ndst1^{-/-} mice (n=5/group) as normalized to HPRT1. The control mice used in this experiment and throughout the study were cre⁻Ndst1^{+/+}. (b) Western analysis of Ndst1 protein in aorta from (1) SM22α-cre⁺Ndst1^{-/-}, (2) control, (3) SMMHC-cre⁺Ndst1^{-/-} and in VSMC from (4) control and (5) SM22α-cre⁺Ndst1^{-/-}. (c) Heparan sulfate disaccharide analysis of Ndst1 deficient mouse models. Bars represent mean ± SE of percent total sulfated, N-sulfated, 2-O sulfated, and 6-O sulfated disaccharides in aortae from control (n = 6), SMMHC-cre⁺Ndst1^{-/-} (n=2) and SM22α-cre⁺Ndst1^{-/-} (n = 3) mice. (d) Schematic representation of a native sulfated heparan sulfate chain and the loss of N-sulfated disaccharides as a result of cre mediated deletion of Ndst1. The heparan sulfate chains are depicted using standard symbol nomenclature. Dashed lines represent the disaccharide units generated by heparinases, which are then separated according to the number and pattern of sulfate groups.

Figure 2.

Heart function is not altered in Ndst1 deficient mice (a) Systolic pressure and (b) LVEDP were not significantly different at baseline in SM22α-cre⁺Ndst1^{-/-} mice (n=4) as compared to control mice (n=4, p=ns). Remaining heart function data is within the text. (d) Medial cell number is significantly reduced in neonatal and adult SM22α-cre⁺Ndst1^{-/-} mice. Bars represent mean ± SE of SMα-actin positive medial nuclei from sections of neonatal femoral artery from control (n=5) and SM22α-cre⁺Ndst1^{-/-} (n=4) and adult femoral arteries from control (n=6) and SM22α-cre⁺Ndst1^{-/-} mice (n=5) (e) Proliferation in neonatal SM22α-cre⁺Ndst1^{-/-} femoral arteries (n=4) compared to control (n=3). Sections were stained with anti-Ki67 and anti-SMα-actin. Bars represent mean ± SE of Ki67 positive VSMC out of total number of VSMC in femoral arteries from both cohorts.

Figure 3.

Loss of Ndst1 decreases intima/media ratio in response to vascular injury in femoral artery in adult mice. (a–c) Representative photomicrographs of femoral arteries 28 days post injury with Verhoeff-Van Gieson staining in control, SMMHC-cre⁺Ndst1^{-/-} and SM22α-cre⁺Ndst1^{-/-} mice. Scale bar = 25 μm. (d) Histogram depicting morphometry measurements of intima/media ratios from control (n=9), SMMHC-cre⁺Ndst1^{-/-} (n=9) and SM22α-cre⁺Ndst1^{-/-} (n=5) femoral arteries 28 days post injury. (e) *In vivo* assessment of cell proliferation by BrdU incorporation. BrdU incorporation was significantly reduced in SM22α-cre⁺Ndst1^{-/-} (n=3) as compared to control mice (n=4) 3 days post vascular injury. (f) *in vitro* inhibition of sulfation decreases proliferation of rat embryonic smooth muscle cells.

Figure 4. (a–c) Representative photomicrographs of femoral arteries 28 days post injury with Masson's Trichrome staining in control, SMMHC-cre⁺Ndst1^{-/-} and SM22α-cre⁺Ndst1^{-/-} mice. Scale bar = 25 μm. (d) Ndst1 deficiency leads to reduced collagen content in the intima in response to injury. Bars represent mean ± SE of collagen content in femoral arteries from control (n=6) SMMHC-cre⁺Ndst1^{-/-} (n=6) and SM22α-cre⁺Ndst1^{-/-} (n=5) mice at 28 days post injury.

Morphometry of femoral arteries from neonatal and adult mice. Measurements were performed on H&E stained sections of femoral arteries (5 sections per mouse) using the NIH ImageJ software. Sections were stained with Verhoeff's Elastin stain for better visualization of EEL and IEL. Values are Mean $\pm$ SE. p values are compared to control.

Table 1

Measurements	Neonatal mice		Adult mice	
	Control (n=5)	SM22a-cre ⁺ Ndst1 ^{-/-} (n=4)	Control (n=6)	SM22a-cre ⁺ Ndst1 ^{-/-} (n=4)
Medial Area (mm ²)	5.847 $\times 10^{-3} \pm 0.001$	3.312 $\times 10^{-3} \pm 0.001$ (<i>p</i> =0.002)	1.462 $\times 10^{-2} \pm 0.008$	1.193 $\times 10^{-2} \pm 0.005$ (<i>p</i> =NS)
Lumen area (mm ²)	3.805 $\times 10^{-3} \pm 0.001$	2.830 $\times 10^{-3} \pm 0.001$ (<i>p</i> =0.005)	3.482 $\times 10^{-2} \pm 0.005$	1.592 $\times 10^{-2} \pm 0.002$ (<i>p</i> =0.02)
IEL (mm)	0.368 ± 0.010	0.280 ± 0.014 (<i>p</i> =0.006)	0.805 ± 0.024	0.659 ± 0.021 (<i>p</i> =0.02)
EEL (mm)	0.390 ± 0.012	0.305 ± 0.013 (<i>p</i> =0.006)	0.889 ± 0.034	0.779 ± 0.021 (<i>p</i> =0.02)

Table 2

Morphometric measurements of femoral arteries in response to injury. 5 μ m sections of femoral arteries at 28 days post injury were stained with H&E and digitized. Sections were stained with Verhoeff's Elastin stain for better visualization of EEL and IEL. All the measurements were performed using NIH ImageJ. Values are mean $\pm$ SE and p values are compared to control.

Measurements	Control (n=9)	SMMHC-cre ⁺ Ndst1 ^{-/-} (n=9)	SM22 α -cre ⁺ Ndst ^{-/-} (n=5)
Lumen area (mm ²)	0.018 $\pm$ 0.002	0.019 $\pm$ 0.002	0.015 $\pm$ 0.003
Intimal area (mm ²)	0.040 $\pm$ 0.003	0.023 $\pm$ 0.002 (<i>p</i> =0.001)	0.024 $\pm$ 0.002 (<i>p</i> =0.006)
Medial area (mm ²)	0.016 $\pm$ 0.001	0.017 $\pm$ 0.001	0.014 $\pm$ 0.001
Intima/Media ratio	2.640 $\pm$ 0.048	1.369 $\pm$ 0.164 (<i>p</i> =0.001)	1.86 $\pm$ 0.197 (<i>p</i> =0.004)
EEL (mm)	0.998 $\pm$ 0.022	0.910 $\pm$ 0.023	0.819 $\pm$ 0.047 (<i>p</i> =0.05)
IEL (mm)	0.898 $\pm$ 0.018	0.829 $\pm$ 0.023	0.724 $\pm$ 0.047 (<i>p</i> =0.001)