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Changes in Expression of Proteoglycan Core Proteins and Heparan Sulfate Enzymes in the Developing and Adult Murine Aorta

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Abstract

Proteoglycan core proteins are linked to four different classes of linear sugar chains referred to as glycosaminoglycans. Heparan sulfate constitutes one of these classes of glycosaminoglycans, and has been shown to be important in developmental processes as well as disease. We designed a low-density gene expression array to identify expression levels of heparan sulfate biosynthetic enzymes and proteoglycan core proteins in the aorta of late stage embryos (E18.5) and adult mice (12 weeks). Significant changes were found in mRNA expression of proteoglycan core proteins syndecan, glypican, decorin, perlecan, and versican from development to adulthood (n=8, p<0.05). Immunohistochemistry revealed a striking localization of both decorin and perlecan staining to the subendothelium in adult vessels, which differed from consistent staining of the endothelium, smooth muscle, and adventitia in development. Significant differences were also identified in the expression of the heparan sulfate modifying enzymes, glururonyl C5 epimerase, 2-O and 6-O sulfotransferases, and N-deacetylase/N sulfotransferases 1–3 (n =8, p<0.05). In conclusion, proteoglycan core proteins and heparan sulfate biosynthetic enzymes in the aorta undergo significant changes in their expression from development to adulthood. These findings may have important biological significance in the specific cell-defined roles of proteoglycan and heparan sulfate related targets in vascular development, maintenance, and response to various perturbations.

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Keywords

Heparan sulfate; Proteoglycan; Gene expression; O-sulfotransferase; N-sulfotransferase

Introduction

Proteoglycan core proteins and the linear sugar chains (glycosaminoglycans) to which they are attached are important for embryonic development, maintenance of cellular function, and response to various stresses [1–3]. Four distinct families of sugar chains bind to proteoglycans: heparan sulfate, chrondroitin sulfate/dermatan sulfate, keratan sulfate, and hyaluronan. The importance of the specific sulfation pattern of the heparan sulfate chains, (regulated by heparan sulfate biosynthetic enzymes) in recruiting and binding growth factors, lipoproteins, and chemokines has just begun to be understood and received particular attention in the vasculature, liver, forebrain and lung [4, 5].

Heparan sulfates are co-receptors for various morphogens and growth factors including those essential for vascular development and maintenance including vascular endothelial growth factor, vascular endothelial growth factor [6], fibroblast growth factor (FGF) [7, 8], and transforming growth factor beta [9]. However, the relative expression pattern of the modification enzymes that regulate heparan sulfate modification and proteoglycan core proteins in the vessel have not been described. In this study, we identified significant differences in both heparan sulfate modifying enzymes as well as proteoglycan core proteins in the developing vs. the adult aorta.

Materials and Methods

Extraction of RNA

Aorta from wild type E18.5 embryos (*n*=8) and 12-week-old adult male mice (*n*=8) were harvested and snap frozen in liquid nitrogen. RNA was harvested from isolated vascular smooth muscle cells (VSMCs) and aorta by the TRizol method (Invitrogen) as previously described [10]. cDNA was synthesized by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Gene Expression

mRNA expression was assessed by designing a customized low density array using a TaqMan Array 384-well fluidic card platform. The gene array included heparan sulfate biosynthetic and degrading enzymes and proteoglycan core proteins (see Table 1 for Taqman Primer IDs). The experiment was replicated, samples were run in duplicate, and expression of each gene was normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase1 (HPRT1). HPRT1 values were not significantly different between embryonic and adult vessels. Analysis was performed on an ABI7900 HT as previously described [10]. Gene expression was analyzed using the delta delta Ct method [11].

Immunohistochemistry

Immunolocalization of perlecan (Hspg2) and decorin (Dcn) was performed by utilizing the vectastain IgG kits according to the manufacturer's instructions (Vector Laboratories). Five micrometer paraffin-embedded sections were stained with anti-perlecan antibody (Santa Cruz Biotechnology) or anti-decorin antibody (R&D Systems). Sections were observed and imaged under ×100 magnification using a Zeiss upright microscope.

Statistical Analysis

Data is represented as mean±SE. ANOVA was performed to analyze the statistical significance in gene expression between embryonic and adult aorta and $p<0.05$ was considered significant.

Results

Proteoglycan Core Proteins

Glypican (Gpc) and syndecan (Sdc) are the two major classes of proteoglycans that bind heparan sulfate. Gpc1, 3, and 4 mRNA expression were significantly higher in embryonic aorta compared to adult aorta (Fig. 1, Table 1). The transcript abundance of Gpc3 was the highest among all its isoforms tested. Sdc1, 2, and 3 mRNA expression was also significantly higher in the embryonic aorta compared to the adult aorta (Fig. 1, Table 1). Sdc4 expression did not change (Fig. 1, Table 1).

Chondroitin sulfate proteoglycans analyzed in this study include Dcn, lumican (Lum), versican (Vcan), aggrecan (Acan), and brevican (Bcan). Dcn is a proteoglycan that bears a single chondroitin/dermatan sulfate chain. Dcn was highly expressed in aorta at E18.5, which decreased significantly in adult mice (Fig. 1). Immunostaining at E18.5 revealed the Dcn staining pattern was strong in the medial and adventitial regions of the vessel as shown in Fig. 2a. In contrast, immunostaining revealed localized expression of Dcn in the subendothelial region of the adult aorta (Fig. 2b). Lum binds to keratan sulfate, and was not detected at any of the time points (Table 1). Both Acan and Vcan expression were significantly higher in the embryonic aorta as compared to adult aorta (Fig. 1, Table 1). Bcan, a proteoglycan mostly found in the CNS was not detected in any cohort (Table 1).

Hspg2, a major proteoglycan of the arterial basement membrane that also binds heparan sulfate, was significantly higher in embryonic aorta compared to adult aorta (Fig. 1, Table 1). Immunostaining of vessels at E18.5 for Hspg2 showed a strong immunostaining throughout the medial wall (Fig. 3a). In comparison, staining the adult vessel showed strongest Hspg2 expression localized to the subendothelial region in adult aorta (Fig. 3b).

Heparan Sulfate Biosynthetic Enzymes

Heparan sulfate biosynthesis occurs in the Golgi complex and involves the participation of several enzymes. Exostosin 1 (Ext1) and exostosin 2 (Ext2) are the co-polymerases that catalyze the elongation of the disaccharides. Ext1 was highly expressed in both the embryonic and adult stages compared to Ext2. No differences in expression of Ext1 and 2 were seen between embryonic and adult aorta (Fig. 4, Table 1).

N-sulfation is the initial step carried out by a family of N-deacetylase/N-sulfotransferases (Ndst1–4). In this study, Ndst1 was the predominant form expressed with a significant decrease in expression from embryonic to adult stage. The expression of Ndst2 and 3 also decreased significantly from embryonic to adult (Fig. 4, Table 1). Ndst4 transcript was undetected (Table 1).

Glucuronyl C5-epimerase (GLCE) catalyzes the epimerization of glucuronic acid to idouronic acid [12]. Expression of GLCE was significantly decreased in adult aorta compared to embryonic vessels (Fig. 4, Table 1). Heparan sulfate 2-O-sulfotransferase (HS2ST), a 2-O sulfotransferase, adds sulfate ion to iduronic acids following C5 epimerization of glucuronic acid to idouronic acid [12] and its transcript decreased significantly in adult aorta as compared to the developing aorta (Fig. 4, Table 1).

Out of the six isoforms of 3-O sulfotransferses, HS3ST1, was the most abundant transcript in the aorta. In contrast, HS3ST2 was undetected in aorta. All the other isoforms were expressed at low levels (Table 1). All three isoforms of heparan sulfate 6-O sulfotransferase (HS6ST) are expressed during embryogenesis with HS6ST1 showing the highest expression. HS6ST3 was undetected (Fig. 4, Table 1).

Ligands to heparan sulfate interactions are also affected by sulfatases and heparanases. There are two sulfatases, sulf1 and 2, that specifically remove sulfate ion present at 6-O position [13]. Transcript abundance of Sulf1 did not change from embryonic to adulthood (Table 1). Sulf2 was undetected. Heparanase (HPSE) is a major mammalian enzyme that degrades heparan sulfate chains [14]. It plays key roles in angiogenesis and cancer metastasis [15]. In the present study, HPSE expression did not differ between embryonic and adult aorta (Table 1).

Discussion

The major findings of this study were that the gene expression and distribution of proteoglycan core proteins changes significantly in the aorta from development to adulthood. With the exception of Sdc4, the transcript abundance of Gpc1–4, Sdc1–3, Acan, Dcn, Hspg2, and Vcan decreased significantly in the adult aorta compared to the late-stage embryo. Immunostaining showed that Dcn and Hspg2 expression in the adult vessels exhibited a gradient-like expression pattern with the strongest expression in the subendothelial space, just beneath the endothelial lining. In contrast, the developing vessels showed ubiquitous expression throughout the medial wall. We focused on these proteins given the high level of expression in the adult as well as the embryo. Wight et al. [16] showed that vascular smooth muscle cells secrete Dcn, which alters the properties of a fibrin matrix. The extent to which this may influence embryonic vascular elasticity vs. adult elasticity is not known. Hspg2 has been shown to inhibit VSMC proliferation [17]. The staining patterns reveal these proteins in a new light and highlight potential differences in a role for each of these core proteins in the vessel during development vs. adulthood.

In the current study, we show that Gpc3 is the most abundantly expressed of the Gpc family. Gpc3 mRNA expression is significantly higher in aortas during the embryonic stage

compared to adulthood. Data from isolated murine VSMC from embryonic and adult aorta showed similar findings (not shown). In humans, loss of function mutations in Gpc3 causes an X-linked disorder known as Simpson–Golabi–Behmel syndrome, a rare congenital disease with severe abnormalities including congenital heart defects Gpc3 knockout mice exhibit excessive coronary arteries in comparison to the number of veins leading to coronary fistulas [18]. More work will be needed to define the cell-specific staining and functional role of Gpc3 in the vasculature.

We also identified a significant decrease in mRNA expression of the transmembrane proteoglycan family members Sdc1, 2, and 3 from embryo to adulthood. This suggests that Sdc1, 2, and 3 may play an important role in vascular development. Sdc1 null mice have been reported to be viable and fertile as well as exhibit normal hair, skin, and ocular surface epithelia [19]. The vasculature in the developing Sdc1 knockout mouse has not been assessed. Sdc1 knockout mouse do exhibit increased intimal lesion and increased VSMC proliferation in response to vascular injury [20]. Sdc1 knockout mouse also exhibit increased endothelial–leukocyte interaction and corneal angiogenesis [21]. Recent work has shown that Sdc1 is critical for migration of stem cells and that this may occur through its interaction with integrin assembly and function [22]. Ablation of Sdc4 leads to degeneration of fetal vessels in the placenta [23]. We did not detect significant differences in expression of Sdc4 in the embryo and adult aortas. However, there was a trend towards an increase in Sdc4 expression in adult aorta. The vascular structure and vascular reactivity of large and small vessels from $Sdc4^{-/-}$ mouse have not been elucidated. Taken together, these functions suggest that the family of Sdcs are important for vascular development and may play a role in VSMC, endothelial and leukocytes. This work provides new evidence showing expression of Sdc1, 2, and 3 is stronger in embryonic vessels than adult. How this translates into biological function and significance is not yet known.

Hspg2 is the major component of the arterial basement membrane and we found that it is significantly expressed in embryonic aorta. In addition, Hspg2 protein was predominantly localized to the subendothelial region of adult aorta, as previously reported [24, 25]. This is significant given the fact that subendothelial proteoglycans including Hspg2 play a major role in the retention of atherogenic lipoproteins [4, 26] and Hspg2 expression has been shown to be upregulated in response to vascular injury [27]. This contrasts from Hspg2 expression in the embryo, which is strong in the medial layer as well as the adventitial layer. These findings suggest that different circulating or local factors may regulate Hspg2 expression in both the embryo and adult. Hspg2 deficiency in mice leads to impaired corneal angiogenesis, delayed wound healing and retarded tumor growth [25]. Tran-Lundmark et al. [26] showed that loss of heparan sulfate chains specifically binding to perlecan in a genetic mouse model resulted in increased atherosclerosis. Thus perlecan and the heparan sulfate chains to which it binds are important in vascular development and disease.

We identified a significant decrease in the chondroitin sulfate family members including Acan and Vcan in adult aorta. Vcan is considered the major chondroitin sulfate of the vessel wall. Vcan is involved in the development and progression of atherosclerosis and restenosis [28] as well as in early cardiogenesis [29, 30]. The role of Vcan and Acan in vessel development has not been explored.

Heparin is perhaps best known in cardiovascular medicine for its role as an anticoagulant in which it binds antithrombin [31]. However, the anticoagulant properties of heparin can be chemically manipulated to either enhance or remove the anticoagulant capacity and uncouple the anticoagulant properties from the anti-inflammatory action of HSPGs [31]. The majority of our understanding of the role of heparan sulfate in vascular injury has come from work demonstrating that heparin inhibits vascular smooth muscle cell proliferation and neointimal formation in injured arteries and that this effect was independent of its anticoagulant capabilities [32–38]. Heparin administration in these studies resulted in a significant loss of bFGF content in the denuded vessel and an increase in the half-life of bFGF in the plasma [35]. Heparin also significantly inhibited proliferation of medial VSMC after denudation [35]. A new generation of heparan sulfate mimetic, PI-88 also inhibited intimal thickening after balloon injury [39]. PI-88 (phosphomannopentaose sulfate) is a 2-kD synthetic polysulfated oligosaccharide that inhibits the activity of the extracellular matrix-degrading enzyme heparanase [39]. In recent years, attention has been focused on the role of heparan sulfate modification in vascular remodeling. Our lab has demonstrated that deletion of the modifying enzyme, Ndst1, in the vessel wall leads to smaller vessels in development and in adulthood and reduced intimal hyperplasia in response to injury [10]. Taken together, these findings suggest that proteoglycan core proteins, heparan sulfate, and chondroitin sulfate play a role in vascular development, as well as remodeling.

In the present study, significant decreases were seen in the expression of Ndst1, 2 and 3 from embryo to adulthood. Of these isoforms, Ndst1 is the most abundant in the embryo and adulthood. Previous work in our lab has identified that knockout of Ndst1 using a smooth muscle specific promoter resulted in smaller vessels as well as defects in vascular remodeling. We documented that a loss of Ndst1 resulted in specific changes in the sulfation pattern of the glycosaminoglycans as measured by a well-characterized and sensitive HPLC technique [10, 40]. In addition, loss of Ndst1 in endothelial cells has been reported to decrease neutrophil trafficking [41]. Finally, MacArthur et al. [5] demonstrated that Ndst1 expression in hepatocytes was critical for the clearance of cholesterol and triglyceride-rich particles.

In this study, we identified a significant decrease in the heparan sulfate-modifying enzyme HS2ST in the adult aorta compared to E18.5. Similar findings were seen in the heparan sulfate-modifying enzyme, GLCE. Mice deficient in HS2ST phenocopy GLCE deficiency and die neonatally due to renal agenesis [42, 43]. Heart and vasculature development however, were reported as unperturbed. HS2ST is also critical for limb bud development [44] and triglyceride clearance [45]. A closer look at the role of HS2ST in vascular smooth muscle cell phenotypes and the role of HS2ST in vascular contractility may be warranted.

Although this study compared over 30 transcripts of major proteoglycan core proteins and modifying enzymes with multiple replicates, a limitation was that this we did not probe all proteoglycans and enzymes. Specifically, neuropillin-1 and betaglycan were not included. The role of these proteoglycans in vascular development has been studied. Neuropilin-1-deficient mice have vascular defects at E8.5 that become progressively more severe [46, 47]. Betaglycan-null mice lack coronary vessels [48, 49]. In the present study, with the exception of Sdc4, the transcript abundance of Gpc1–4, Sdc1–3, Acan, Dcn, Hspg2, and Vcan

decreased significantly in the adult aorta compared to the late-stage embryo. This may be related to the composition of the cell types in the aorta. The staining patterns of both Dcn and Hspg2 suggest that the increased expression in the aorta is not due to a robust change in expression in the smooth muscle cells or the single layer of endothelium but rather from the cell types that make up the adventitia and/or any cells that were present in the lumen. Further studies are needed to fully understand the biological relevance of these changes in cardiovascular development and disease.

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Abbreviations

Acan	aggrecan
Dcn	decorin
Gpc1	glypican 1
Gpc3	glypican 3
Gpc4	glypican 4
Hspg2	heparan sulfate proteglycan 2 (perlecan)
Sdc1	syndecan 1
Sdc2	syndecan 2
Sdc3	syndecan 3
Sdc4	syndecan 4
Vcan	versican
Bcan	Brevican
Lum	Lumican
Ext1	exostosin 1
Ext2	exostosin 2
HS2ST	heparan sulfate 2-O-sulfotransferase
HS3ST1	heparan sulfate 3-O-sulfotransferase 1
HS3ST2	heparan sulfate 3-O-sulfotransferase 2
HS3ST3A1	heparan sulfate 3-O-sulfotransferase 3A1
HS3ST3B1	heparan sulfate 3-O-sulfotransferase 3B1
HS3ST6	heparan sulfate 3-O-sulfotransferase 6
HS6ST1	heparan sulfate 6-O-sulfotransferase 1
HS6ST2	heparan sulfate 6-O-sulfotransferase-2

HS6ST3	heparan sulfate 6-O-sulfotransferase 3
GLCE	glucuronyl C5-epimerase
Ndst1	N-deacetylase/N-sulfotransferase 1
Ndst2	N-deacetylase/N-sulfotransferase 2
Ndst3	N-deacetylase/N-sulfotransferase 3
Ndst4	N-deacetylase/N-sulfotransferase 4
Sulf1	sulfatase 1
Sulf2	sulfatase 2
HPSE	heparanase
VSMC	vascular smooth muscle cell
FGF	fibroblast growth factor
HPRT1	hypoxanthine phosphoribosyl transferase1

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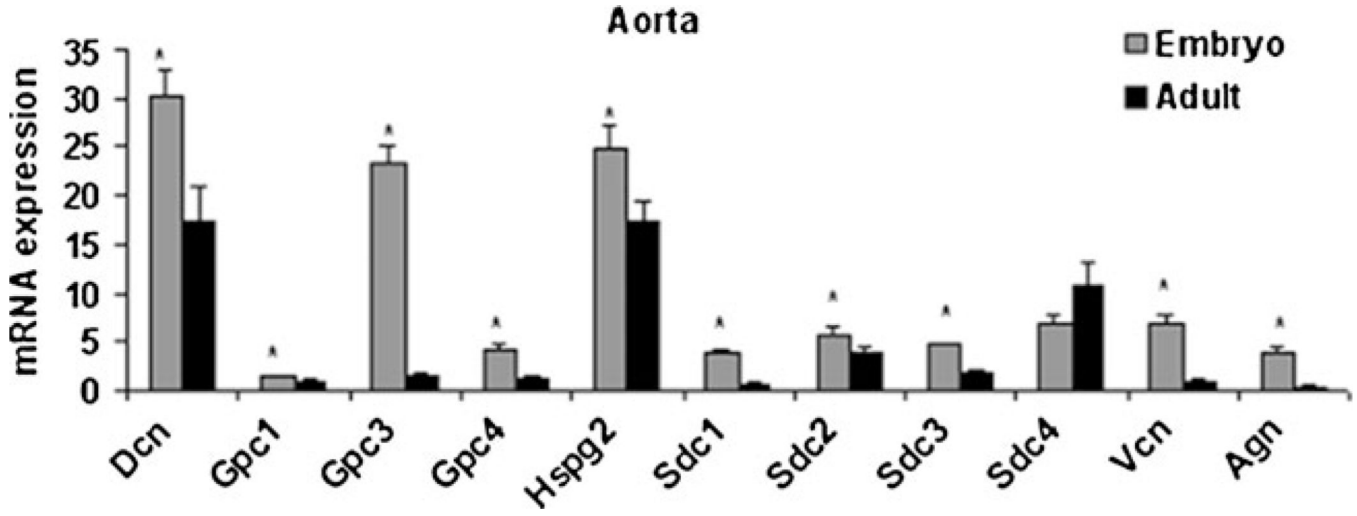

Fig. 1.

mRNA expression of proteoglycan core proteins in aorta from late-stage embryos and adult male mice (n=8/ group, p<0.05)

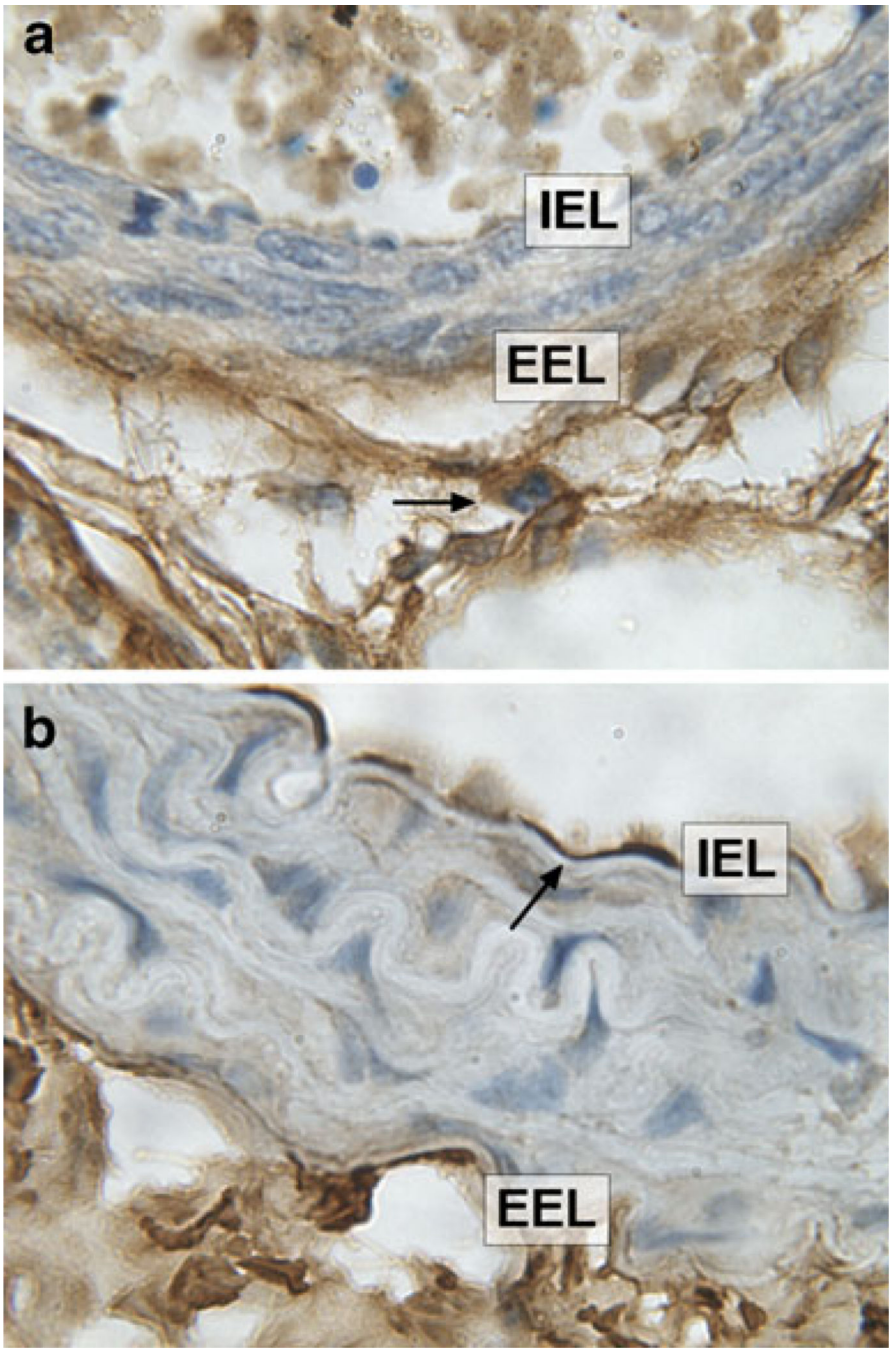

Fig. 2.

a Representative photomicrographs of E18.5 vessels showing Dcn staining in the medial and adventitial region (*arrowhead*) in aorta. **b** In contrast, Dcn staining was found predominantly in the subendothelial region (arrowhead) in adult aorta. Magnification ×100. *IEL* internal elastic lamina, *EEL* external elastic lamina

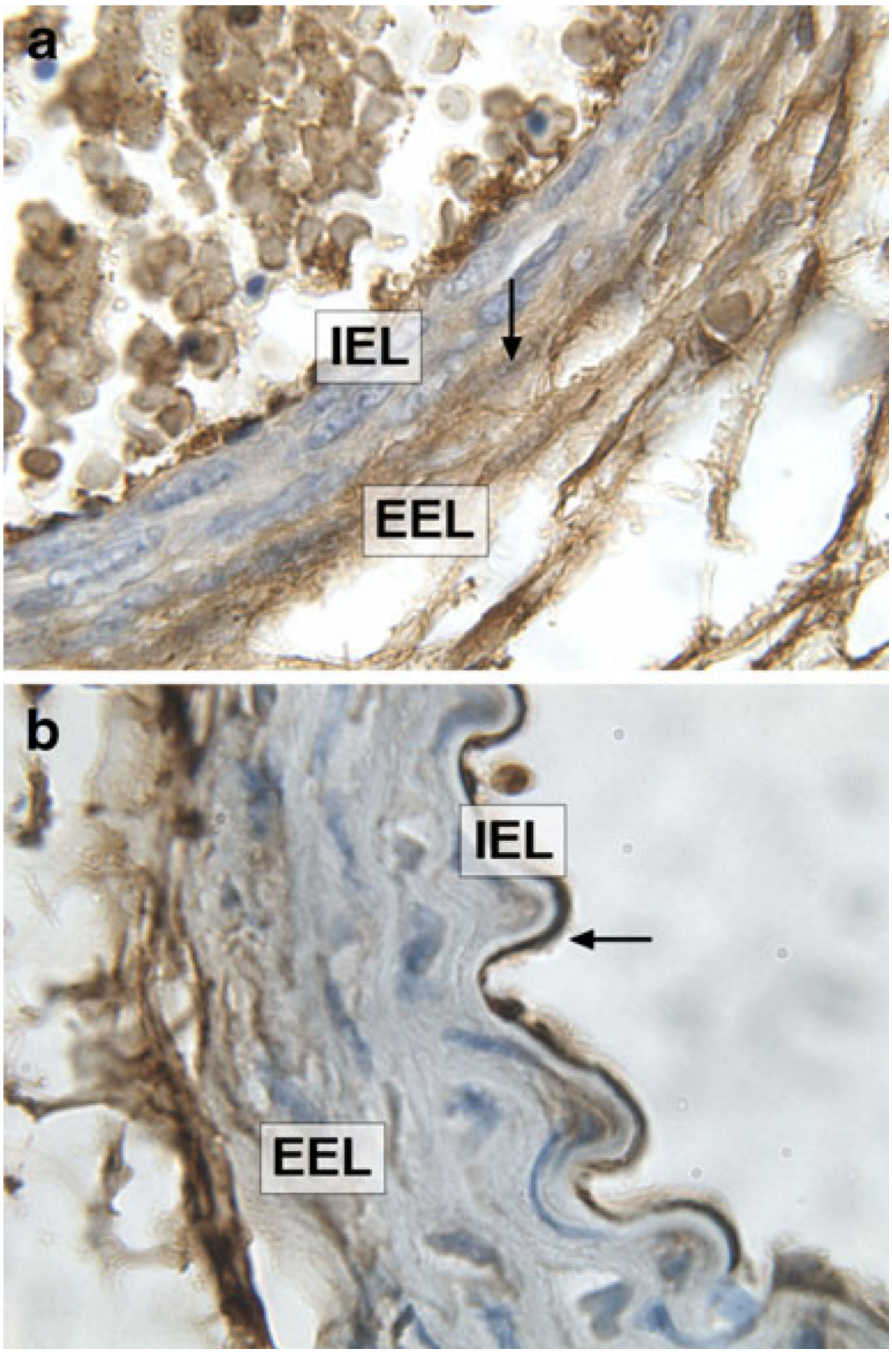

Fig. 3.

a Representative photomicrographs showing Hspg2 staining in the medial (*arrowhead*) and adventitial region in aorta from E18.5 embryos **b** and predominantly in the subendothelial region (*arrowhead*) in aorta from adult male mice. Magnification ×100. *IEL* internal elastic lamina, *EEL* external elastic lamina

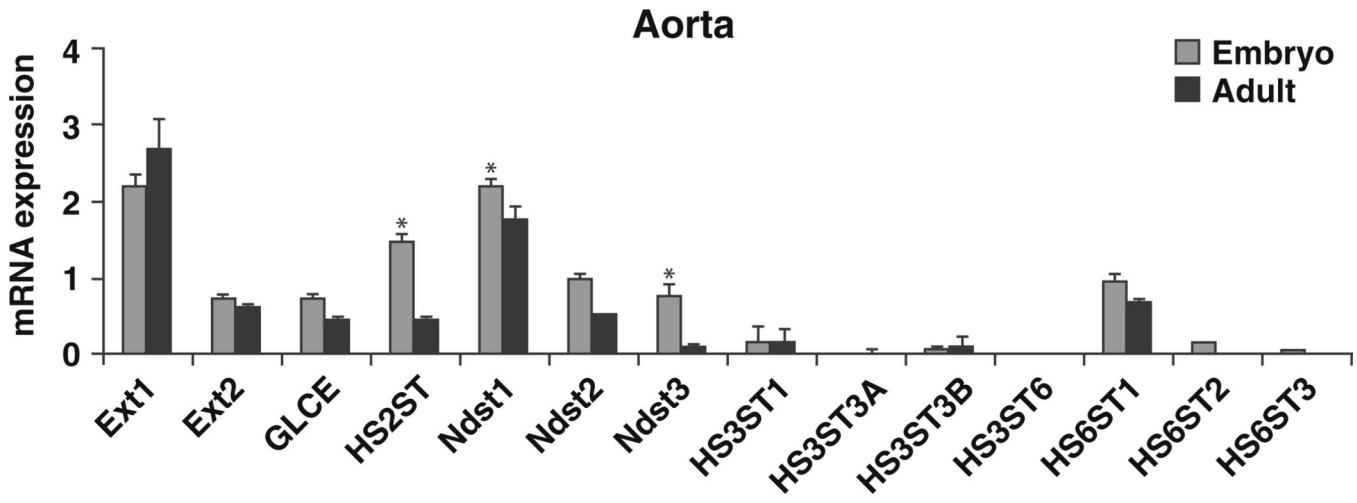

Fig. 4.

mRNA expression of heparan sulfate biosynthetic enzymes in aorta from embryos and adult male mice (n=8/ group, p<0.05)

Table 1

Gene abbreviation, Taqman primer ID on the low density array, normalized delta Ct values for RNA extracted from aortas of late stage embryo and adult C57BL6 mice and significance

Gene	Primer ID	Expression Embryo (*n*=8)	Adult (*n*=8)	*P* value
Gpc1	Mm00497305_m1	1.33±0.11	1.02±0.09	0.04
Gpc3	Mm00516722_m1	23.36±1.99	1.44±0.21	0.001
Gpc4	Mm00515035_m1	4.11 ±0.46	1.17±0.10	0.001
Sdc1	Mm00448918_m1	3.84±0.40	0.53±0.08	0.001
Sdc2	Mm00484718_m1	5.73±0.68	3.68±0.60	0.04
Sdc3	Mm01179831_m1	4.62±0.31	1.56±0.25	0.001
Sdc4	Mm00488527_m1	6.84±0.77	10.91 ±2.21	ns
Hspg2	Mm01181165_m1	24.94±2.39	17.28±1.97	0.03
Dcn	Mm00514535_m1	30.34±2.70	17.34±3.71	0.01
Acan	Mm00545807_m1	3.88±0.58	0.28±0.10	0.001
Vcan	Mm01283063_m1	6.93±0.65	0.89±0.16	0.001
Bcan	Mm00476090_m1	ND	ND	ND
Lum	Mm00500510_m1	ND	ND	ND
Ext1	Mm00468769_m1	2.19±0.18	2.68±0.39	ns
Ext2	Mm00468775_m1	0.71 ±0.07	0.61±0.04	ns
Ndst1	Mm00447005_m1	2.18±0.11	1.77±0.16	0.05
Ndst2	Mm00447818_m1	0.99±0.08	0.52±0.02	0.001
Ndst3	Mm00453178_m1	0.78±0.14	0.12±0.04	0.001
Ndst4	Mm00480767_m1	ND	ND	ND
GLCE	Mm00473667_m1	0.73±0.06	0.46±0.04	0.002
HS2ST	Mm00478684_m1	1.46±0.13	0.47±0.04	0.001
HS3ST1	Mm01964038_s1	0.20±0.01	0.18±0.05	ns
HS3ST2	Mm00616933_m1	ND	ND	ND
HS3ST3A1	Mm00780907_s1	0.03±0.01	0.03±0.01	ns
HS3ST3B1	Mm00479621_m1	0.08±0.01	0.11 ±0.03	ns
HS3ST6	Mm01299930_m1	ND	ND	ND
HS6ST1	Mm01229698_s1	0.94±0.11	0.71±0.02	ns
HS6ST2	Mm00479296_m1	0.16±0.02	0.02±0.01	0.001
HS6ST3	Mm00479297_m1	ND	ND	ND
Sulf1	Mm00552283_m1	4.58±0.43	4.00±1.07	ns
Sulf2	Mm00511193_m1	ND	ND	ND
HPSE	Mm00461768_m1	0.22±0.03	0.22±0.04	ns

Acan aggrecanl; *Dcn* decorin; *Gpc1* glypican1; *Gpc3* glypican3; *Gpc4* glypican 4; *Hspg2* heparan sulfate proteoglycan 2 (perlecan); *Sdc1* syndecan 1; *Sdc2* syndecan 2; *Sdc3* syndecan 3; *Sdc4* syndecan 4; *Vcan* versican; *Lum* lumican; *Bcan* brevican; *Ext1* exostoses 1; *Ext2* exostoses 2; *H2ST1* heparan sulfate 2-O-sulfotransferase 1; *HS3ST1* heparan sulfate 3-O-sulfotransferase; *HS3ST3A1* heparan sulfate 3-O-sulfotransferase 3A1; *HS3ST3B1* heparan sulfate, 3-O-sulfotransferase 3B1; *HS3ST6* heparan sulfate 3-O-sulfotransferase 6; *HS6ST1* heparan sulfate 6-O-sulfotransferase 1; *HS6ST2* heparan sulfate 6-O-sulfotransferase-2; *HS6ST3* heparan sulfate 6-O-sulfotransferase 3; *GLCE* glucuronyl C5-epimerase; *Ndst1* N-deacetylase/N-sulfotransferase 1; *Ndst2* N-deacetylase/N-sulfotransferase 2; *Ndst3* N-deacetylase/N-sulfotransferase 3; *Ndst4* N-deacetylase/N-sulfotransferase 4; *Sulf1* sulfatase 1; *Sulf2* sulfatase 2; *HPSE* heparanase; *ND* not determined; *ns* not significant