Functional Overlap Between Chondroitin and Heparan Sulfate Proteoglycans During VEGF-Induced Sprouting Angiogenesis

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Objective—Heparan sulfate proteoglycans regulate key steps of blood vessel formation. The present study was undertaken to investigate if there is a functional overlap between heparan sulfate proteoglycans and chondroitin sulfate proteoglycans during sprouting angiogenesis.

Methods and Results—Using cultures of genetically engineered mouse embryonic stem cells, we show that angiogenic sprouting occurs also in the absence of heparan sulfate biosynthesis. Cells unable to produce heparan sulfate instead increase their production of chondroitin sulfate that binds key angiogenic growth factors such as vascular endothelial growth factor A, TGFβ, and platelet-derived growth factor B. Lack of heparan sulfate proteoglycan production however leads to increased pericyte numbers and reduced adhesion of pericytes to nascent sprouts, likely due to dysregulation of TGFβ and platelet-derived growth factor B signal transduction.

Conclusion—The present study provides direct evidence for a previously undefined functional overlap between chondroitin sulfate proteoglycans and heparan sulfate proteoglycans during sprouting angiogenesis. Our findings provide information relevant for potential future drug design efforts that involve targeting of proteoglycans in the vasculature. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: angiogenesis ● biology, developmental ● endothelium ● glycosaminoglycan ● vascular biology

Angiogenesis, the formation of new blood vessels from preexisting vasculature, occurs during several pathological conditions such as rheumatoid arthritis and cancer.1 Sprouting angiogenesis, a major mechanism of neovascularization, depends on a set of spatially and temporally strictly regulated processes, including (1) induction and selection of a leading tip cell among endothelial cells (ECs); (2) tip cell migration followed by stalk formation; (3) lumen formation and vessel anastomosis, and finally (4) stabilization of the immature vessel through the recruitment of mural cells (pericytes).2 Key factors identified to regulate sprouting angiogenesis include vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), and platelet-derived growth factor B (PDGFB). Importantly, proteoglycans carrying heparan sulfate (HS) polysaccharide chains have been shown to bind these and other angiogenic modulators through their HS chains and act as coreceptors. HSPGs thereby support VEGFA- and FGF2-signaling required for angiogenesis, as well as the formation of PDGFB gradients to allow for recruitment of pericytes along nascent vessels.3–10

HS biosynthesis is strictly regulated resulting in the formation of a multitude of sulfated protein-binding epitopes. The HS chains evolve by the stepwise addition of alternating glucuronic acid and N-acetylgalactosamine residues by the HS polymerases exostosin 1 and 2 (EXT1 and EXT2) to oligosaccharide acceptor linkage regions attached to core proteins. As the HS chains grow in length, they undergo several consecutive modification steps, including N-deacytlation and N-sulfation of alternating N-acetylgalactosamine residues by N-deacytalyse/N-sulfotransferases (NDSTs), epimerization of alternating glucuronic acid to iduronic acid by a C5-epimerase, and finally sulfation at various locations by O-sulfotransferases. It is important to note that although protein–protein interactions rely on unique primary sequences, HS–protein interactions depend on HS charge distribution as well as overall conformation,11 rather than on a specific sequence of sulfated disaccharide units.12 From this notion it follows that other sulfated polysaccharides such as chondroitin sulfate (CS), having a charge density similar to...
by gel chromatography on Sephadex G50 (0.5 × 100 cm) eluted in 0.2 mol/L NaCl; fractions of 0.4 mL were collected and the $^{35}$S-radioactivity measured.

**Compositional Analysis of HS and CS**

Unlabeled HS and CS were isolated from ESCs and EBs as described in the online-only Data Supplement, and thereafter analyzed by RP-HPLC according to the protocols previously described by Ledin et al.21

**Isolation of Endothelial Cells From EBs and Quantitative RT-PCR**

Protocols for isolation of ECs on basis of CD31 expression, together with protocols for quantitative RT-PCR, are described in the online-only Data Supplement.

**Immunofluorescence, Western Blotting, and Signaling Assays**

Whole EBs in collagen gel were cut out and fixed in 4% p-formaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature, and thereafter blocked and permeabilized for 1 hour. The fixed EBs were incubated overnight at 4°C with primary antibodies, followed by washing and incubation with secondary antibodies the next day. Detailed procedures for analysis by immunofluorescence, Western blotting, and growth factor signaling assays are described in the online-only Data Supplement.

**Nitrocellulose Filter Binding Assay**

$^{35}$S-Labeled GAGs were purified from wt and Ext1$^{-/-}$ ESCs and EBs (stimulated with VEGFA for 12 days) and probed for binding to VEGFA-165, bFGF, and TGFβ1 using the previously described nitrocellulose filter-binding assay.22

**Results**

**Ext1$^{-/-}$ EBs Are Capable of Sprouting Angiogenesis, Albeit With Defective Pericyte Attachment**

The EB model for sprouting angiogenesis was used to study sprout formation when HS biosynthesis was eliminated or deficient in both ECs and mural cells. EBs were generated from genetically engineered mouse ESCs deficient in the HS-polymerase Ext1 or lacking the HS-modifying enzymes Ndst1 and NDST2. Whereas the Ext1$^{-/-}$ ESCs have been shown to lack HS biosynthesis, Ndst1$^{-/-}$ ESCs synthesize an undersulfated HS containing only low levels of 6-O-sulfate groups.19

EBs were grown in a 3-dimensional matrix of collagen I and stimulated with VEGFA165 to enhance sprouting angiogenesis.23 Both wt and Ext1$^{-/-}$ EBs showed vigorous angiogenic sprouting in response to VEGFA at day 12 of differentiation (Figure 1A and 1D). Indeed, no significant difference in sprout area fraction was observed between wt and Ext1$^{-/-}$ EBs (Figure 1B, 1E, and 1I), suggesting that HS is not required for early angiogenic sprouting and tube formation. However, the Ext1$^{-/-}$ EBs exhibited increased pericyte formation, accompanied by an increased occurrence of detached pericytes, as compared to the wt control (Figure 1C, 1F, and 1J–1K). In contrast, Ndst1$^{-/-}$ EBs failed to produce angiogenic sprouts during the first 12 days of culture, in agreement with previous findings (Figure 1G),9 whereas culture for 15 days or longer supported some formation of angiogenic sprouts in the Ndst1$^{-/-}$ EBs. No pericytes were however detected along the Ndst1$^{-/-}$ sprouts, indicating suppressed pericyte formation (Figure 1H).
Aberrant TGFβ- and PDGFB-Signaling in Ext1−/− EBs

TGFβ and PDGFB have been shown to regulate pericyte differentiation and attachment.24,25 In agreement with the above described pericyte defects we found that both the TGFβ- and PDGFB-signaling pathways were affected in the Ext1−/− EBs. Markedly higher levels of phospho-Smad2 (a downstream target of TGFβ) were observed without addition of exogenous TGFβ in Ext1−/− EBs compared to the wt control, and the phospho-Smad2 levels did not increase further by acute TGFβ stimulation (Figure 1L and 1M). We next used the in situ proximity ligation assay to determine whether PDGF-receptor beta (PDGFRβ) was expressed and activated in the different cultures. As shown in Figure IA in the online-only Data Supplement, PDGFRβ was indeed expressed and tyrosine phosphorylated in both wt and Ext1−/− pericytes.

Next, PDGFRβ was immunoprecipitated to analyze the degree of tyrosine phosphorylation per receptor in response to PDGFB at different time points after stimulation. A higher degree of PDGFRβ phosphorylation was detected in Ext1−/− EBs as compared to wt EBs in all samples analyzed (Figure 1N and 1O and Figure IIA in the online-only Data Supplement). Quantitative analysis of PDGFRβ protein expression was performed by immunoblotting of EB lysates, and the results showed that Ext1−/− EBs express ~50% higher levels of PDGFRβ compared to wt EBs (Figure IIB in the online-only Data Supplement). Further, although not statistically significant, a tendency for decreased phosphorylation of the
downstream target AKT, and increased phosphorylation of ERK1/2 was noted in the Ext1−/− EBs (Figure IIC in the online-only Data Supplement). Adding either exogenous PDGFB or TGFβ1 to the Ext1−/− cultures did not rescue pericyte attachment. Instead, these growth factor treatments clearly increased both the pericyte numbers and the extent of pericyte detachment (Figure IB in the online-only Data Supplement). Even though TGFβ- and PDGFB-signaling appeared dysregulated in Ext1−/− cultures, VEGFR2 activation was shown to be similar in VEGFA165-treated wt and Ext1−/− EBs (data not shown) in agreement with the strong VEGFA-induced sprouting seen in these cultures (Figure 1D–1F).

Differentiated Cells in Ext1−/− EBs Are Incapable of HS Biosynthesis

Several aspects of angiogenesis and VEGF function have been shown to be regulated by HS.26 The fact that Ext1−/− EBs formed vessels in response to VEGFA could still in theory be due to some residual HS biosynthesis during differentiation in these cultures, despite the lack of EXT1 expression. To address this issue, we isolated metabolically 35S-labeled GAGs (including both HS and CS) from wt and Ext1−/− ESCs and EBs and performed GAG structural analysis.

Starting with ESCs, treatment of the total GAG pool from wt stem cells with CSase ABC, which selectively cleaves CS but not HS, resulted in degradation of approximately 50% of the material (Figure 2A). In contrast, the total GAG pool isolated from Ext1−/− ESCs were completely degraded by CSase treatment, indicating that no HS was produced by the Ext1−/− ESCs, in full agreement with previous observations16 (Figure 2A). The same experiment was repeated with labeled GAGs from differentiated EBs with vascular structures. Consistently, half of the GAG pool isolated from wt cells was degraded by CSase (Figure 2B). A small fraction of GAGs isolated from Ext1−/− EBs however appeared to be resistant to CSase treatment (Figure 2B). A second round of enzyme treatment nevertheless reduced the amount of intact GAGs substantially, indicating that incomplete degradation at least in part could explain the small amount of nondegraded 35S-macromolecules in the Ext1−/− EB GAG preparation (Figure 2B). To exclude that HS was synthesized by the Ext1−/− EBs, the EB-derived GAG preparations were treated with nitrous acid at pH 1.5 which cleaves HS at N-sulfated residues. All GAGs isolated from Ext1−/− EBs resisted this treatment, whereas approximately 50% of GAGs isolated from wt EBs was degraded (Figure 2C). It could thus be concluded that Ext1−/− EBs, just like Ext1−/− ESCs, produce CS but not HS.

Ext1−/− EBs Exhibit Increased CS Biosynthesis

As Ext1−/− EBs were unable to produce HS but still formed angiogenic sprouts, we hypothesized that CS functionally could compensate for the lack of HS. The total amounts of CS and HS synthesized by ESCs and angiogenic EBs of the different genotypes were therefore determined. Both HS and CS synthesis were increased during the course of differentiation in wt cells, although there was a decrease in the amount of both types of GAGs in Ndslt2−/− EBs compared to ESCs (Figure 3A and 3B). Accordingly, Ndslt2−/− EBs may be regarded as null mutants for both HS and CS biosynthesis, providing an explanation for the markedly delayed formation of sprouts in these cultures (Figure 1G and 1H). The levels of
HS produced by the Ext1\textsuperscript{−/−} ESCs or EBs were at the limit of detection (Figure 3A) and therefore considered to be insignificant background signals, in agreement with the data presented in Figure 2. Notably, the total amounts of CS recovered were twice as high in Ext1\textsuperscript{−/−} ESCs and EBs as compared to wt cells (Figure 3B). The detailed sulfation patterns of wt and Ext1\textsuperscript{−/−}-derived CS revealed that in both cases the majority of CS disaccharide units were 4-O-sulfated. Also, there was no substantial difference between wt and Ext1\textsuperscript{−/−} cells with regard to overall degree or pattern of sulfation of CS, as determined by disaccharide analysis (Figure 3C and Figure III in the online-only Data Supplement). Taken together, analysis of HS and CS produced by wt and Ext1\textsuperscript{−/−} cells at different stages of differentiation showed that the loss of HS in vascularized Ext1\textsuperscript{−/−} EB is compensated by a 2-fold increase in CS production.

Figure 3. Exostosin 1 (Ext1)\textsuperscript{−/−} cells show increased production of chondroitin sulfate (CS) and elevated levels of decorin and NG2 mRNA. Total amounts of heparan sulfate (HS) produced by wildtype (wt), N-deacetylase/N-sulfotransferases (Ndst1/2)\textsuperscript{−/−} and exostosin 1 (Ext1)\textsuperscript{−/−} embryonic stem cells (ESCs) and embryoid bodies (EBs) quantified using the RPIT-HPLC method (A). Quantification of CS produced by wt, Ndst1/2\textsuperscript{−/−}, and Ext1\textsuperscript{−/−} ESCs (B). The overall sulfate content of CS (C), specified according to type of substituent as total 2-O-sulfated (2S), total 6-O-sulfated (6S), and total 4-O-sulfated disaccharides (4S), demonstrate increased production of CS in Ext1\textsuperscript{−/−} cultures, albeit with normal degree of sulfation. The average of 2 separate experiments is shown. Quantitative RT-PCR analysis of the CSPGs endocan (D), decorin (E), and NG2 (F) after isolation of CD31\textsuperscript{+} endothelial cells (ECs) from wt and Ext1\textsuperscript{−/−} EBs. EC indicates CD31\textsuperscript{+} cells; NEC, nonendothelial (CD31\textsuperscript{−}) cells.
**Elevated Decorin and NG2 mRNA Levels in Ext1<sup>−/−</sup> EBs**

The expression levels of core proteins and enzymes involved in CS biosynthesis were initially analyzed in ECs as well as in non-ECs isolated from both Ext1<sup>−/−</sup> and wt EBs using a qPCR screen (Figure IV in the online-only Data Supplement and Table I in the online-only Data Supplement, and data not shown). Validation of the expression of selected genes was thereafter performed by additional qPCR analyses (Figure 3D–3F). Endocan, known to carry CS, was found to be equally expressed in CD31-positive ECs isolated from VEGFA-induced wt and Ext1<sup>−/−</sup> EBs, whereas no endocan mRNA was detected in the non-EC fraction (CD31-negative) (Figure 3D). In contrast, the expression levels of the CS-bearing proteoglycan core proteins decorin and NG2 were increased more than 5- and 10-fold, respectively, in non-ECs isolated from Ext1<sup>−/−</sup> EBs as compared to wt (Figure 3E and 3F). Other genes linked to CS biosynthesis were expressed at equal levels in Ext1<sup>−/−</sup> and wt cultures (data not shown). These results demonstrate that the increased production of CS in Ext1<sup>−/−</sup> cultures is accompanied by increased expression of proteoglycan core proteins.

**CS Interacts With and Supports PDGFB- and TGFβ-Signaling**

A standard nitrocellulose filter binding assay was used to test interactions between Ext1<sup>−/−</sup>-derived CS and the angiogenic growth factors TGFβ1, PDGFB, and VEGFA165. All 3 growth factors were shown to bind metabolically 35S-labeled CS isolated from wt and Ext1<sup>−/−</sup> EBs (Figure 4A). We next analyzed signaling pathways in ECs (HUVECs) or smooth muscle cells (hAoSMCs) of human origin treated or not by CSase ABC or heparitinase III prior to growth factor stimulation, to degrade extracellular CS and HS. Figure V in the online-only Data Supplement shows that cell surface HS/CS degradation was efficient under these conditions. Phosphorylation of VEGFR2 and activation of downstream targets in HUVECs, as well as PDGFB signaling in hAoSMCs, were not significantly disturbed by degradation of GAGs under these conditions (Figure 5B–4D and Figure VIA in the online-only Data Supplement). In contrast, treatment of hAoSMCs with CS-degrading enzymes before TGFβ stimulation significantly decreased the level of Smad2 phosphorylation by approximately 25% (Figure 4E and 4F); degradation of HS was without significant effects. Admittedly, the here performed enzymatic degradation of extracellular HS and CS, although efficient, is likely incomplete, providing an explanation for the small effects recorded on VEGFA- and PDGFB-signaling in HUVECs and hAoSMCs, respectively. We therefore turned to the Ndst1<sup>−/−</sup>/Ndst1/2<sup>−/−</sup> mouse embryonic fibroblast (MEF) model. Abramsson and coworkers have previously shown that PDGFB signaling in these cells, expressing normal levels of CS, is reduced due to reduced levels of functional HS. Using the Ndst1<sup>−/−</sup>-MEF model, we could show that PDGFB signaling could be further reduced by degradation of CS by CSase treatment (Figure 4G and Figure VIB in the online-only Data Supplement), providing additional evidence for the involvement of CS in angiogenic growth factor signaling.

**Discussion**

The present study demonstrates for the first time that angiogenic sprouting can occur in the absence of HS biosynthesis. Ext1<sup>−/−</sup> EBs, unable to synthesize HS but exhibiting increased CS biosynthesis, were accordingly shown to form vascular sprouts in response to VEGFA. The main morphological phenotypes detected in the Ext1<sup>−/−</sup> condition were increased formation of pericytes, accompanied by an increased extent of pericyte detachment from the vessel sprouts. Sprout formation in Ndst1/2<sup>−/−</sup> EBs was, on the other hand, severely compromised, and pericytes did not form in these cultures. This result is in agreement with the finding that differentiated Ndst1/2<sup>−/−</sup> EBs are effective “double knockouts” for both HS and CS (Figure 3). Thus, although sprout formation indeed is dependent on GAG function, our data suggest that selective loss of HS production in part can be compensated by increased CS biosynthesis. The fact that the CS-producing Ext1<sup>−/−</sup> EBs but not the GAG-null Ndst1/2<sup>−/−</sup> EBs supported angiogenesis strongly argue for a functional overlap between HS and CS in sprouting angiogenesis (Figure 5). Importantly, these results challenge the view that HS is strictly required for all steps of the angiogenic process, and strengthen the notion that there is a broad selectivity for interactions between protein ligands and equally sulfated, yet distinct, HS and CS epitopes.

It is outside the scope of this study to identify the mechanism behind the increased CS biosynthesis in Ext1<sup>−/−</sup> EBs. However, HS and CS biosynthesis share some common substrates (UDP-sugars and the sulfate donor PAPS), and utilize the same tetrasaccharide linkage region for initiation of biosynthesis onto core proteins. A reduction in HS biosynthesis will thus likely result in more available substrates for CS biosynthesis, providing an explanation to the increased production of CS in Ext1<sup>−/−</sup> EBs.

The defect in pericyte attachment found in Ext1<sup>−/−</sup> EBs was mirrored at the level of TGFβ signaling and PDGFRβ phosphorylation. A trend was also seen for altered activation of AKT and ERK1/2 in response to PDGFB in Ext1<sup>−/−</sup> EBs. Dysregulated TGFβ and PDGFB signaling (Figure 1) offer highly plausible mechanisms for the observed loss in pericyte attachment in Ext1<sup>−/−</sup> EBs. TGFβ signaling has previously been shown to promote pericyte differentiation, and the increased TGFβ signaling may therefore explain the higher pericyte number in the Ext1<sup>−/−</sup> EBs. The Ext1<sup>−/−</sup> phenotype was not rescued by the addition of exogenous PDGFB or TGFβ1. Instead, both the number of pericytes and the extent of detachment were further increased by the exogenous addition of these growth factors (Figure IB in the online-only Data Supplement), emphasizing the need for regulated presentation of these factors, likely in the form of properly shaped and maintained extracellular concentration gradients.

The CS produced by the Ext1<sup>−/−</sup> EBs was capable of binding TGFβ, PDGFB, and VEGFA. Notably, the expression levels of CS biosynthetic enzymes were unchanged in these cultures, although the amounts of some of the CS-modified core proteins, eg, decorin and NG2 were increased, which may be linked to the increased production of CS. Decorin belongs to the family of small leucine rich proteoglycans and has been shown to regulate extracellular matrix...
organization as well as growth factor signaling. Indeed, decorin binds to PDGFB and may inhibit PDGFB-stimulated vascular smooth muscle cell function in a dose-dependent manner. Interestingly, the active form of TGFβ is known to mediate the de novo induction of pericytes from the mesenchymal cell lineage during embryonic development and also binds to decorin in the extracellular matrix. In addition, decorin has been shown to inhibit or stimulate angiogenesis dependent on the biological context.

Our data furthermore suggest that CS is required for proper TGFβ signaling. The mechanisms underlying the sensitivity to loss of CS displayed in our analyses of TGFβ signaling is unclear (Figure 4). However, the fact that CSase treatment reduced TGFβ/TGFβR signaling in hAoSMCs suggests that CS could be engaged in ternary complexes together with growth factors and receptors to promote receptor complex assembly. Interestingly, TGFβ receptor III or betaglycan is a “promiscuous” proteoglycan that has the capacity to carry either HS chains or CS chains, both HS and CS at the same time, or no GAG chains at all. The betaglycan protein core has further been shown to bind TGFβ and to act as a TGFβ coreceptor by facilitating TGFβ receptor I/ TGFβ receptor III complex formation. The chemical nature of the GAG influences TGFβ receptor III activity, but the exact role of the different GAGs in TGFβ receptor III function is still not well understood. We suggest that disturbed balance between HS and CS production (as seen in Ext1/EBs) impacts the GAG composition of TGFβ receptor III and consequently alters signaling. Finally, using the Ndst1/MEF model, we could show that PDGFB signaling in a genetic background where HS biosynthesis but not CS biosynthesis is suppressed could be further reduced by CSase treatment (Figure 4G), providing evidence for a modulatory role of CS in PDGFB signal transduction.

Pathological conditions characterized by vessel immaturity, vessel excess, or dysregulated growth factor production, are often accompanied by pericyte detachment. Pericyte
detachment or other types of pericyte deficiencies have been shown to lead to increased vessel tortuosity and leakage, as well as to the formation of microaneurysms, with an increased risk for bleedings. Interestingly, metastatic spread of cancer may be facilitated by vessel instability at least in part due to the lack of attached pericytes. Here, EXT1-deficiency and decreased HS production was accompanied by pericyte detachment from angiogenic sprouts. PDGFβ signaling was decreased in the Ext1−/− EBs, which expressed increased levels of CS. We suggest that CS-dependent elevation of TGFβ signaling (Figure 1) may indirectly affect the cellular response to PDGF.

It is tempting to speculate that the observed functional overlap between HS and CS may be relevant not only to angiogenic sprouting, but also to other complex morphogenetic processes influenced by GAG-binding growth factors. Tampering with HS biosynthesis could represent a possible way to target HS–protein interactions of pathophysiological significance. Efforts are indeed ongoing to evaluate GAG-mimetics or inhibition of HS biosynthesis for therapeutic purposes. However, the partial functional overlap between HSPGs and CSPGs demonstrated by the present study suggests that targeting of either HS or CS production alone may not be sufficient to efficiently inhibit angiogenesis. Further, inhibition of HS depending on strategy may also affect CS biosynthesis, as seen here with the 2-fold increase in CS biosynthesis in Ext1−/− EBs. Finally, simultaneous blocking of both HS and CS biosynthesis would probably also lead to severe side effects. At any rate, the close link between HS and CS biosynthesis, as well as their overlapping functions, should be kept in mind when considering future drug strategies to influence GAG-modulated biological processes.

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References


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Supplemental Material

Supplemental Materials and Methods

Culture of stem cells and EBs
Embryonic stem cells (ESCs) were routinely grown on mouse embryonic fibroblasts in DMEM with glutaMAX (Invitrogen), 25mM HEPES, 1.2mM sodium pyruvate, 19mM monothioglycerol (Sigma), 15% fetal bovine serum, and 1000 units/ml of leukemia inhibitory factor (LIF). EBs were generated as previously described\(^1\). Briefly, at day 0, 1200 ESCs were aggregated in hanging drops (20 µl) without LIF and VEGFA. After 4 days, EBs were seeded either in 10 cm tissue culture dishes (for PDGFRß signaling assays), in T75 flasks (for metabolic labeling), in T225 flasks (for HS and CS compositional analysis) or into a gel of collagen I (for analysis of angiogenic sprouting by immunofluorescence), in the presence or absence of 30 ng/ml recombinant VEGFA\(_{165}\) (PreproTech). In rescue experiment, PDGFB (20 ng/ml or 50 ng/ml) and TGFß1 (2 ng/ml or 5 ng/ml) both obtained from Preprotech were added to VEGFA-induced Ext1\(^{-/-}\) cultures between day 8 and day 12. The EBs were normally harvested day 12 unless indicated otherwise.

Isolation of metabolically labeled HS and CS
After a 20 h incubation of ESCs or EBs with \(^{35}\text{S}-\text{sulfate}, \text{the cell media was discarded, and the cells washed four times with cold phosphate-buffered saline followed by incubation in 2 ml of solubilization buffer (50 mM Tris-HCl, pH 8, 1% Triton X-100, 0.10 M NaCl), containing the protease inhibitors EDTA, phenylmethanesulfonyl fluoride, and pepstatin A at 4°C for 1 h. After centrifugation at 13000 x g for 10 min,
the proteoglycans were isolated from the solubilized cell lysate on a 0.3-ml column of DEAE-Sephacel (GE Healthcare Biosciences), equilibrated in 50 mM Tris-HCl pH 8, 0.1% Triton X-100, 0.1 M NaCl. The column was washed first with equilibration buffer and subsequently with 50 mM acetate buffer, pH 4.0 containing 0.1% Triton X-100 and 0.1 M NaCl. The proteoglycans were eluted with 50 mM acetate buffer, pH 4.0, containing 0.1% Triton X-100 and 2 M NaCl and alkali-treated as described2.

After desalting in water on PD10 columns (GE Healthcare Biosciences) followed by lyophilization, the 35S-labeled GAGs were subjected to enzymatic or chemical degradation, see the main text.

Isolation of HS and CS for compositional analysis by RPIP-HPLC

Cells or EBs were dissolved in 0.5 ml of Pronase buffer (50 mM Tris/HCl, pH 8.0, 1% Triton X-100, 1 mM CaCl2, 0.8 mg/ml Pronase) and incubated end-over-end for 19 h at 55 °C. After heat inactivation of the enzyme, MgCl2 was added to a final concentration of 2 mM. After addition of Benzonase (12 milliunits), the sample was incubated for 2 h at 37 °C followed by heat inactivation. The NaCl concentration was subsequently adjusted to 0.1 M and the sample centrifuged at 13,000 x g for 10 min. The supernatant was diluted with 0.5 ml 50 mM Tris/HCl pH 8.0, 0.1 M NaCl, and applied to a Sep-Pak C18 cartridge (Waters) that had been primed first with methanol, then with water and finally with 50 mM Tris/HCl pH 8.0, 0.1 M NaCl. The cartridge was washed with 2 ml of the Tris/HCl buffer and the washing fraction combined with the flow through fraction and applied to a 0.2 ml DEAE-Sephacel column equilibrated in loading buffer (50 mM Tris/HCl pH 8, 0.1% Triton X-100, 0.1 M NaCl). After washing with six column volumes of loading buffer, six volumes of low pH buffer (50 mM NaAc, pH 4.0, 0.1% TritonX-100, 0.1 M NaCl) and six
volumes of loading buffer without Triton X-100, the GAGs were eluted with 0.6 ml of elution buffer (50 mM Tris/HCl, pH 8.0, 1.5 M NaCl). The samples were next desalted using NAP-10 columns (GE Healthcare Biosciences) equilibrated in water, and dried by SpeedVac centrifugation. The GAG pool was digested with 50 milliunits of CSase ABC in 100 µl 40 mM Tris/Ac buffer pH 8.0. The CS digestion was allowed to proceed for 4 h at 37 °C and the sample was then boiled to stop the reaction. After removal of 10 µl for CS analysis by RPIP-HPLC as previously described, HS was recovered after a second round of DEAE-Sephacel chromatography as described for total GAG isolation. Purified HS was dissolved in 200 µl of heparinase buffer (5 mM Hepes buffer, pH 7.0, 50 mM NaCl, 1 mM CaCl₂) and divided into two equal aliquots. One of the aliquots was treated with 0.4 milliunits each of heparitinases I, II and III and incubated for 16 h at 37 °C. The other aliquot (control sample) was incubated under the same conditions without enzymes. After heat inactivation the samples were analyzed by RPIP-HPLC as described by Ledin and co-workers.

**Isolation of endothelial cells from EBs and quantitative RT-PCR**

Sheep anti-rat IgG Dynabeads (Dynal, Invitrogen) were coated with rat anti-mouse CD31 (BD Pharmingen) and incubated with collagenase-treated wild type or Ext1−/− EBs for 30 min at 4°C. The CD31+ and CD31- cell populations were collected according to the manufacturers instructions for RNA isolation using Qiazol lysis buffer (Qiagen). Samples were subjected to reverse transcription (Superscript III, Invitrogen) and amplified using intron spanning primers to CSPGs and CS biosynthesis enzymes. Quantitation was done by the 2∆∆CT method, using β-actin as control RNA. CT values from triplicate assays were used to calculate fold-expression as compared to β-actin. The results shown in Fig. 3 are based on three independent
assays, and the mean values ± s.d. are shown. The statistical significance was determined using ANOVA. See Supplemental Table 1 for information about the primers used to detect PG core protein transcripts.

**Immunofluorescence**

At day 12 of differentiation, whole EBs in collagen gel were cut out and fixed in 4% p-formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, and thereafter blocked and permeabilized in 3% BSA, 0.1% Tween20 in PBS for one h. EBs were incubated overnight at 4°C with primary antibodies: rat anti-mouse CD31 antibody (Pharmingen) and rabbit polyclonal anti-NG2 antibody (AB5320, Chemicon International). Secondary antibodies used were: Alexa 488 goat anti-rat IgG and Alexa 555 goat anti-rabbit IgG (Molecular Probes). Samples were analyzed in a Zeiss Axiovert 200M microscope equipped with a Zeiss AxioCam MRm camera and pictures were taken using Axiovision software (Zeiss). Quantification of the morphology and localization of NG2+ cells in the different cultures was scored in a blind fashion. Pictures were taken at 20X magnification (11 EBs for each genotype; in total 420 cells were scored in wt and 1100 cells in Ext1−/− EBs). Results are presented as mean ± s.d. and the statistical significance was determined using the ANOVA test.

hAoSMCs were stained for CS using mouse monoclonal anti-CS antibody (Sigma; clone CS-56), and HUVECs were stained for degraded HS or sulfated HS using the 3G10 or HepSS1 antibodies respectively (both from Seigakaku).

**Western blotting and signaling assays**

Smad2, PDGFRβ and VEGFR2 phosphorylation and downstream target activation of PDGFRβ in response to TGFβ1, PDFGB or VEGFA165 stimulation respectively
were analyzed in Ext1−/− and wt EBs cultured in regular cell culture dishes from day 4 to 11 in complete medium. At day 11, the cultures were starved in 0.5% serum for 16 hs before TGFβ1, PDGF or VEGFA165 (PeproTech) was added to give the final concentration of 5, 100 or 50 ng/ml respectively. All EBs were harvested and corresponding cell lysates prepared as previously described 1.

HUVECs and hAoSMCs were starved in 1% serum for 16 hours, and thereafter treated with heparitinase III (1U/ml) or CSase ABC (25mU/ml) for 1.5 h, thereafter rinsed 3 times with starvation media and stimulated with VEGFA165 (100 ng/ml; 5 min), TGFβ1 (5 ng/ml; 1h) or PDGFB (50 ng/ml; 5 min) prior to harvest for analysis using Western blotting. For immunoprecipitation experiments, the cell lysates were incubated with polyclonal anti-PDGFRβ (sc-432, Santa Cruz Biotechnologies) or anti-VEGFR2 (R&D, AF644 and AF357) antibodies for 2 hs at 4°C, followed by incubation with Immunosorb A beads (Medicago) at 4°C for 45 min. Proteins were released by boiling the beads in sample buffer (Tris-HCl pH 6.8, 1.5% SDS, 4.35% glycerol, 4% β-mercaptoethanol, 0.0025% bromophenol blue). Immunoprecipitates or total cell lysates were separated on SDS polyacrylamide gel and transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare Biosciences). The membranes were incubated with the following primary antibodies: rabbit anti-phospho Smad2 (antibody kindly provided by Dr Aris Moustakas, the Ludwig Institute for Cancer Research, Uppsala, Sweden), mouse anti-Smad1/2/3 (sc-7960; Santa Cruz), mouse anti-phosphotyrosine (4G10; Upstate, Lake Placid, NY), rabbit polyclonal anti-PDGFRβ antibody (sc-432; Santa Cruz), goat anti-human VEGFR2 (AF357; R&D Systems), goat anti-mouse VEGFR2 (AF644; R&D Systems), rabbit anti-phospho Akt, rabbit anti-phospho-ERK1/2, rabbit anti-total Akt or rabbit anti-total ERK1/2 (all from Cell Signaling Technology). Immuno-reactivity
was visualized by enhanced chemiluminescence (ECL or ECL Plus Western Blotting System, GE Healthcare Biosciences). Ndst1−/− MEFs 5 were grown in high-glucose DMEM with 10% FBS, starved for 16 hrs in DMEM supplemented with 0.2 BSA and treated with CSase (75 mU/ml, 3 x 1 h incubations) prior to stimulation with PDGFB (100 ng/ml, 10 min) followed by analysis of cell lysates by Western blotting. All cell signaling experiments were repeated at least 3 times, representative blots are shown. The total levels of PDGFRβ in total EB lysates were normalized to the level of β-actin (detected by rabbit anti-β-actin antibody (968227; Abcam) in each sample. Quantified results obtained by Western blotting are presented as the mean value ± s.d. for the indicated number of experiments. The statistical significance was determined using the Student’s t-test.

**In Situ PLA Analysis of phospho-PDGFRβ in EBs**

wt and Ext1−/− EBs were cultured for 14 days on glass slides in presence of VEGF (30 ng/ml). At day 14, the EBs were starved for 20 hs and then stimulated or not with PDGFB (100 ng/ml) for 1 h on ice. The samples were fixed using ethanol and thereafter blocked in 20% goat serum (Invitrogen) containing 2.5 ng/µl sonicated salmon sperm DNA (GE Healthcare Biosciences), 2.5 mM L-cysteine (Sigma), 0.1% Tween 20, 5 mM EDTA in PBS for 2 h at 37 °C. The following primary antibodies were applied and incubated overnight at 4 °C: mouse anti-phosphotyrosine (anti-pY100; #9411, Cell Signaling; 1: 250 final concentration) and rabbit anti-PDGFRβ (#3169, Cell Signaling; 2 ng/µl final concentration) dissolved in blocking buffer. Incubation with secondary proximity probes (4.9 ng/µl anti-mouse proximity probe and 1.6 ng/µl anti-rabbit proximity probe; Olink Biosciences AB, Uppsala, Sweden), hybridization, ligation, rolling circle amplification and detection were performed as
previously described with one major modification; prior to the detection step, the samples were incubated overnight at 4°C with rat anti-CD31 and rabbit anti-NG2 antibodies. In the detection step secondary antibody anti-rat Alexa-633 (Invitrogen) and anti-rabbit FITC Fab2-fragment (Jackson ImmunoResearch) were added. Z-stack images were collected using a LSM-500 confocal microscope (Zeiss) and converted to maximum intensity projections using ImageJ software (NIH). For better visualization, the signals (so called “blobs”) were enhanced with a maximum filter (0.5 pixel radius) and the red channel subtracted from the green and blue channels before merging.

Supplemental figure legends

Supplemental Figure I. Phosphorylation of PDGFRβ in situ. (A) Phosphorylation of PDGFRβ was analyzed in situ on wt and Ext1−/− EBs using the proximity ligation assay. Co-staining for CD31 (blue) and NG2 (green) was used to identify ECs and PCs respectively. Each red dot corresponds to one detected molecule of phosphorylated PDGFRβ. Red dots located outside NG2+ cells correspond to activated PDGFRβ in fibroblasts (counterstaining for fibroblasts not shown). (B) Addition of PDGFB or TGFβ1 to Ext1−/− cultures from day 8 to day 12 did not rescue the pericyte attachment phenotypes, but instead increased both the pericyte numbers and the extent of pericyte detachment.

Supplemental Figure II. PDGFB signaling in Ext1−/− EBs. (A) wt and Ext1−/− EBs were at day 12 stimulated with PDGFB for the indicated time periods. PDGFRβ
protein was immunoprecipitated (IP), followed by immunodetection of phosphotyrosine residues (P-Tyr), and for PDGFRβ. See the graph in Fig. 1O for a quantification of tyrosine-phosphorylated PDGFRβ relative to total PDGFRβ protein \( (n = 2) \). (B) Total cell lysates from wt and Ext1\(^{−/−}\) EBs were analyzed for PDGFRβ expression by immunoblotting. The levels of PDGFRβ was normalized to the levels of β-actin in each sample \( (n = 3) \), a representative blot is shown in the top panel. (C) The ability of PDGFB to induce PDGFRβ downstream signaling was studied in wt and Ext1\(^{−/−}\) EBs by immunoblotting of total lysates as indicated. Quantifications of the results are shown in the two lower panels \( (n = 4) \).

**Supplemental Figure III. Composition of chondroitin sulfate from wt and Ext1\(^{−/−}\) stem cells and EBs.** CS was isolated and digested with CSase and the disaccharide products analyzed by RPIP-HPLC. 0S, \( Δ\text{Hexuronic acid} \) (HexA)-GalNAc; 4S, \( Δ\text{HexA}-\text{GalNAc4S} \); 6S, \( Δ\text{HexA}-\text{GalNAc6S} \); 2S, \( Δ\text{HexA2S}-\text{GalNAc} \); 6S4S, \( Δ\text{HexA}-\text{GalNAc4S} \); 4S2S, \( Δ\text{HexA2S}-\text{GalNAc4S} \); 6S4S2S, \( Δ\text{HexA2S}-\text{GalNAc4S6S} \). Note that the 0S disaccharide pool also may contain disaccharides derived from hyaluronan (Hya) that was not completely removed during purification.

**Supplemental Figure IV. qPCR screen to identify altered PG core protein mRNA expression in Ext1\(^{−/−}\) EBs as compared to wt EBs.** VEGFA-stimulated EBs of the respective genotype were harvested at day 12 of culture. Mechanical and enzymatic digestion of EBs was followed by isolation of CD31+ endothelial cells using magnetic Dynabeads coated with anti-CD31 antibodies. Thereafter total RNA from the endothelial cell fraction (A) and the left over cell fraction (B) was isolated and analyzed by qPCR to detect the mRNA levels of a set of PG core proteins known
to carry CS. See Supplemental Table I for the full gene names corresponding to the gene symbols shown here.

**Supplemental Figure V. Verification of HEPase and CSase activity.** The levels of HS in HUVECs (A) and CS in hAoSMCs (B) are reduced by heparitinase III (HEPase) and Chondroitinase ABC (CSase) treatments respectively. HUVECs were stained for neoepitopes generated by HEPase digestion using the 3G10 antibody, or stained for the presence of sulfated HS using the HepSS1 antibody. hAoSMCs were stained for CS using the CS-56 antibody.

**Supplemental Figure VI. Effects of HEPase and CSase treatment on VEGFA and PDGFB signaling.** (A) Activation of VEGFR2 in HUVECs as judged by phosphorylation of the receptor tyrosine residue 1175 and ERK1/2 phosphorylation was not significantly affected by treatment with HEPase or CSase. (B) Western blot analysis showing the activation of PDGFRβ by PDGFB in Ndst1−/− MEFs with impaired HS production. Here, treatment with CSase leads to reduced receptor phosphorylation (n = 3, representative blots are shown).

**Supplemental Table**

**Supplemental Table I. List of primers used to detect the expression levels of PG core proteins in Ext1−/− and wt EB cultures.**
References


Supplemental Figure I, Le Jan et al.

A

CD31/NG2/phospho-PDGFRβ (in situ PLA)

wt EB day 12

Ext1−/− EB day 12

- PDGFB  + PDGFB

B

CTRL  +PDGFB 20 ng/ml  + PDGFB 50 ng/ml  +TGFB1 2 ng/ml  +TGFB1 5 ng/ml

Ext1−/− EB day 12

CD31/NG2
Supplemental Figure II, Le Jan et al.

A

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**IP: PDGFRβ**

P-Tyr

PDGFRβ

B

**Fold difference total PDGFRβ protein**

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\[ p = 0.6 \times 10^{-3} \]

n = 3

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**Time (min) after PDGFB stim.**

**Total AKT**

**Total ERK1/2**

**n = 4**

**Fold induction P-AKT**

**Fold induction P-ERK**
Supplemental Figure III, Le Jan et al.

Proportion of total disaccharides (%)

WT ESC
WT EB day 12
Ext<sup>1<sup>–</sup></sup> ESC
Ext<sup>1<sup>–</sup></sup> EB day 12
Supplemental Figure V, Le Jan et al.

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Supplemental Figure VI, Le Jan et al.

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