

## **Soluble perlecan domain i enhances vascular endothelial growth factor-165 activity and receptor phosphorylation in human bone marrow endothelial cells**

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### **Abstract**

**Background:** Immobilized recombinant perlecan domain I (PlnDI) binds and modulates the activity of heparin-binding growth factors, in vitro. However, activities for PlnDI, in solution, have not been reported. In this study, we assessed the ability of soluble forms to modulate vascular endothelial growth factor-165 (VEGF165) enhanced capillary tube-like formation, and VEGF receptor-2 phosphorylation of human bone marrow endothelial cells, in vitro.

**Results:** In solution, PlnDI binds VEGF165 in a heparan sulfate and pH dependent manner. Capillary tube-like formation is enhanced by exogenous PlnDI; however, PlnDI/VEGF165 mixtures combine to enhance formation beyond that stimulated by either PlnDI or VEGF165 alone. PlnDI also stimulates VEGF receptor-2 phosphorylation, and mixtures of PlnDI/VEGF165 reduce the time required for peak VEGF receptor-2 phosphorylation (Tyr-951), and increase Akt phosphorylation. PlnDI binds both immobilized neuropilin-1 and VEGF receptor-2, but has a greater affinity for neuropilin-1. PlnDI binding to neuropilin-1, but not to VEGF receptor-2 is dependent upon the heparan sulfate chains adorning PlnDI. Interestingly, the presence of VEGF165 but not VEGF121 significantly enhances PlnDI binding to Neuropilin-1 and VEGF receptor-2.

**Conclusions:** Our observations suggest soluble forms of PlnDI are biologically active. Moreover, PlnDI heparan sulfate chains alone or together with VEGF165 can enhance VEGFR-2 signaling and angiogenic events, in vitro. We propose PlnDI liberated during basement membrane or extracellular matrix turnover may have

similar activities, in vivo.

## **Background**

Perlecan, a heparan sulfate proteoglycan with preferred localization to vascular basement membranes, is comprised of a ~480 kDa protein core with five distinct domains (I - V). Domains II-V share structural homologies with other protein modules [1]. In contrast, N-terminal domain I (PlnDI) is structurally unique. As a ~22 kDa protein core, PlnDI contains 172 amino acid residues that give rise to a sperm protein, enterokinase and agrin (SEA) module localized downstream of three Ser-Asp-Gly motifs that serve as glycosaminoglycan (GAG) attachment sites [2,3].

Through the chondroitin and heparan sulfate GAG chains attached to domain I, perlecan functions as a ligand reservoir for storage, release, and protection of heparin-binding growth factors (reviewed by Whitelock et al., 2008). These interactions allow perlecan to modulate a range of biological functions, including angiogenesis (reviewed by Bix and Iozzo, 2008)[4]. Recent studies suggest immobilized forms of perlecan and PlnDI bind VEGF165 to coordinate developmental angiogenesis by modulating VEGF165/VEGFR-2 signaling [5,6]. However, a role for soluble forms of PlnDI and the mechanism(s) by which it modulates VEGF165/VEGFR-2 signaling is unclear.

Angiogenic activities of VEGFs are mediated primarily through two receptors [7], VEGFR-1 or fms-like tyrosine kinase 1 [8] and VEGFR-2, also known as kinase domain receptor, and fetal liver kinase 1 [9,10]. Although VEGFR-1 exhibits higher binding affinity for VEGFs, VEGFR-2 dominates VEGF induced mitogenic and angiogenic responses on endothelial cells [11,12]. VEGFR-2 signaling is enhanced by interactions with co-receptors such as heparin/heparan sulfate and Neuropilin 1 (NRP-1) [13]. In addition, VEGF binding to VEGFR-2 and NRP-1 is enhanced by exogenous heparin [14,15]. Although the natural cell surface and basement membrane polysaccharide, in vivo, is heparan sulfate, not heparin, few cell surface or extracellular HSPGs have been shown to modulate VEGF/VEGFR interactions [6,16].

Herein, we tested the hypothesis that soluble forms of recombinant PlnDI bind and increase VEGF165/VEGFR-2 interactions on human bone marrow endothelial cells, in vitro. Observations from this investigation suggests soluble forms of recombinant PlnDI are biologically active and capable of interacting with components of the VEGFR-2 signaling complex, enhance activity and downstream signaling related to

endothelial cell angio-genic processes.

## Results

### Purification and biochemical characterization of PlnDI

Recombinant PlnDI was purified from conditioned media of HEK 293 EBNA clones as reported previously [17], and further enriched by passage through a Sephar-ose CL-6B column. This additional step removed high molecular weight contaminants secreted into the serum free media (i.e., full length perlecan). Aliquots of the eluted product were subsequently analyzed by SDS-PAGE and Western blotting to identify the GAG chain composition and preparation purity.

In Coomassie blue stained SDS-PAGE gels, undigested samples displayed a broad band between ~45-117 kDa (Figure 1A, lane 1); whereas aliquots pre-treated with a hepari-nase cocktail yielded a distinct band at ~36 kDa, with a broad band between 55 -71 kDa (Figure 1A, lane 2). Chon-droitinase ABC pre-digestion yielded a distinct band at ~33 kDa and broad band between 45 -117 kDa (Figure 1A, lane 3). Pre-digestion with both GAG lyases yielded a single band at 33 kDa (Figure 1A, arrow lane 4). The additional bands appearing in Figure 1A, lanes 2-4, represent BSA (j, ~66 kDa), chondroitinase ABC ( $\delta$ , ~100 kDa), and hepari-nases I (a, ~43 kDa), II (b, ~84 kDa), and III (g, ~70 kDa).

In Alcian blue stained SDS-PAGE gels, undigested samples displayed a broad band between ~45-117 kDa

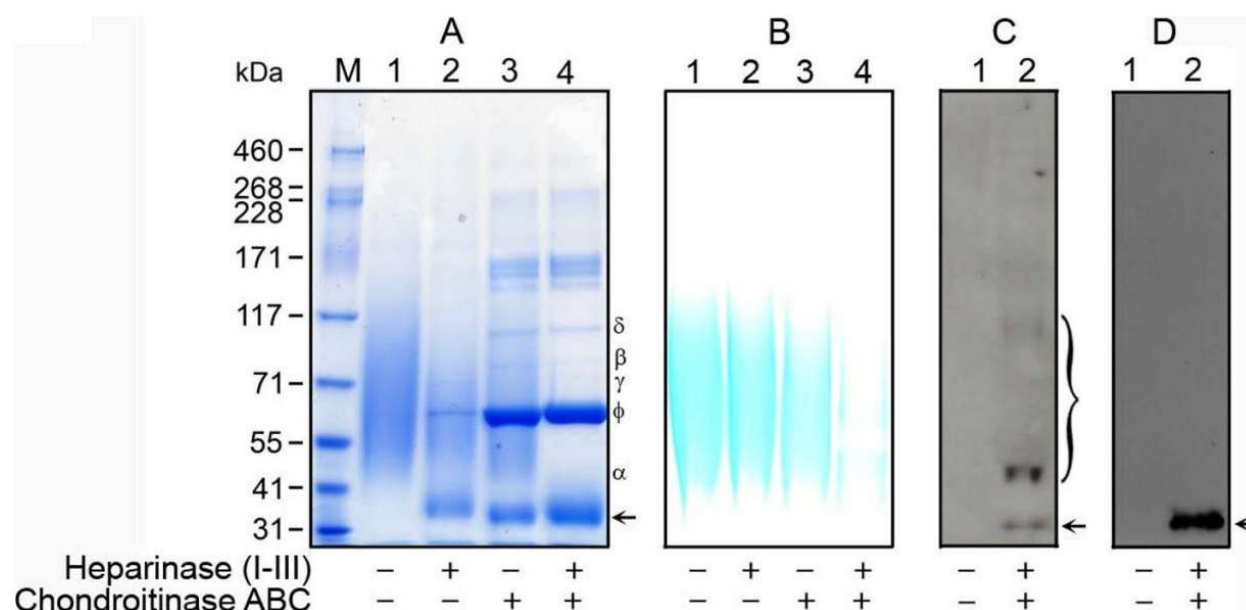


Figure 1 SDS-PAGE and Western blot analysis of PlnDI. (A) Coomassie blue staining; (B) Alcian blue staining: lane 1, undigested PlnDI; lane 2, heparinase cocktail treated PlnDI; lane 3, chondroitinase ABC treated PlnDI; lane 4 heparinase cocktail and chondroitinase ABC treated PlnDI. (C) Western blot analysis with PlnDI and (D) heparan sulfate stub (3G10) specific antibodies respectively: Lane 1, undigested PlnDI; lane 2, heparinase cocktail and chondroitinase ABC treated PlnDI. Heparinase cocktail is a mixture of heparinases I, II, and III. Arrows indicate the protein core of PlnDI released after heparinase cocktail and chondroitinase ABC digestion. a,b,g,δ, j indicate the migration positions of heparinases I, II, III, chondroitinase ABC, and BSA respectively. Bracket in panel C denotes immunoreactive products released following incomplete digestion.

(Figure 1B, lane). Aliquots pre-treated with a heparinase cocktail yielded a broad band between ~50-100 kDa (Figure 1B, lane 2). Chondroitinase ABC pre-digestion yielded a broad band between ~50-84 kDa (Figure 1B, lane 3). Pre-digestion with both GAG lyases abolished the majority staining.

**The presence of PlnDI was confirmed by Western blotting** using anti-PlnDI specific antibodies (CSI-0071) and antibodies (3G10) to anti-Δ-heparan sulfate that recognize heparan sulfate neo-epitopes, generated following heparinase cleavage (arrow Figure 1C and 1D). Neither antibody recognized undigested products; however, anti-PlnDI antibodies recognized partially digested products (bracket in Figure 1C, lane 2) and both antibodies recognize a distinct band at 33 kDa (arrow, Figure 1C and 1D). The 33 kDa band reflects the domain I core protein adorned with GAG chain linkage residues following heparinase digestion.

Biochemical analysis of PlnDI suggests a protein and uronic acid content of 49% and 37%, respectively (Table 1). Hexosamine (monosaccharide) compositional analysis revealed PlnDI GAGs are composed predominantly of galactosamine (60%) relative to glucosamine (40%) (Table 1). The disaccharide composition of purified PlnDI revealed 6-sulfated disaccharide as the major Δdi-CS with lesser amounts of nonsulfated and 4-sulfated disaccharides (Table 2). The major Δdi-HS derived from PlnDI was nonsulfated and Δdi-S1 with considerable, but lesser amounts of Δdi-S2, -6-sulfated, -N-sulfated, and -triS disaccharides (Table 2). The HS GAG chains on PlnDI contain approximately 3 fold more 6-O- than 2-O-sulfation.

#### **VEGF165 binds to PlnDI in a heparan sulfate dependent manner**

To identify requirement(s) for VEGF165 binding to PlnDI, both solid and solution phase binding assays were performed. In solid phase binding assays, immobilized PlnDI binds VEGF165 in a heparan sulfate dependent

manner (Figure 2).

Heparinase cocktail treatment of PlnDI, prior to immobilization on nitrocellulose, reduced VEGF165 binding by ~75% (Figure 2). In contrast, pre-digestion with chondroitinase ABC did not alter VEGF165 binding. Studies with the PlnDI protein core, prepared following digestion with a mixture of both enzymes, suggest VEGF165 poorly binds this region. VEGF antibodies do not bind immobilized PlnDI (Figure

**Table 1 Biochemical composition of PlnDI**

| Sample          | Composition (% dry weight) |             | Hexosamine (%) |      |
|-----------------|----------------------------|-------------|----------------|------|
|                 | Protein                    | Uronic acid | GalN           | GlcN |
| PlnDI<br>(0.88) | 49 (0.37)                  | 37 (0.29)   | 60 (0.87)      | 40   |

- 2). In competitive inhibition assays, heparin [0.25 µg/ml] prevented ~80% of VEGF165 binding to PlnDI (Figure 2). In solution, requirements for VEGF165 binding to PlnDI were similar, but the capacity of binding demonstrated pH dependence (Figure 3A). When the pH of solution was reduced from 8.0 to 7.0 then 6.0, VEGF165 binding was reduced by 50% and 80%, respectively (Figure 3A). To identify VEGF165 specific binding, the background binding of VEGF165 to nitrocellulose was subtracted from total bound to PlnDI [18]. Employing this approach, PlnDI-HS chains account for nearly all VEGF165 binding, and the presence of CS chains masks VEGF165 interaction with HS (Figure 3B). In panel B, neutral pH was chosen to more closely reflect tissue culture conditions of subsequent experiments.

## PlnDI modulation of VEGF165 bio-activity

To identify a role for PlnDI in modulating VEGF165 activity in vitro, human bone marrow endothelial cells were employed in two independent assays: 1) VEGF165-enhanced capillary tube-like formation; 2) VEGF165 -enhanced phosphorylation of VEGFR-2. In capillary tube-like formation assays, the ability of bone marrow endothelial cells to form tube-like structures in the presence of exogenous VEGF165 +/- PlnDI was quantified. Under serum free conditions, the addition of soluble VEGF165 (positive control) and PlnDI demonstrated dose dependent increases in lengths of tube-like structures formed (Figure 4A-B and 1F). Optimal concentrations for VEGF165 [20 ng/ml] and PlnDI [12.5 µg/ml] increased tube-like formation 35% and 24%, respectively.

Studies employing PlnDI, pre-treated with either chondroitinase ABC and/or a heparinase cocktail suggests the ability of PlnDI to enhance tube-like formation is HS chain dependent (Figure 4C). Moreover, PlnDI activity is further enhanced when its CS chains are removed. Interestingly, PlnDI/VEGF165 mixtures combine to enhance tube-like formation 16% relative to VEGF165 alone (Figure 4D). The synergy between PlnDI and VEGF165 is PlnDI-HS chain dependent (Figure 4D). PlnDI protein core/VEGF165 mixtures produce tube-like structures indifferent from those by VEGF165 alone. Unexpectedly, heparin/VEGF165 mixtures do not synergize in this system (Figure 4E).

Since the presence of endogenous cell surface HS complicates the studies above, experiments employing bone marrow endothelial cells without cell surface HS were performed. Under these conditions, VEGF165 and PlnDI enhance tube-like formation (Figure 5); however, PlnDI/VEGF165 mixtures did not combine to further enhance the lengths of tube-like structures. Subsequent dose response studies suggested elevated concentrations of VEGF165 and PlnDI are required for maximal activity. Increasing PlnDI concentration two fold [25.0 µg/ml]

**Table 2 Disaccharide composition of Chondroitin and Heparan sulfate chains of PlnDI**

| Sample | Chondroitin sulfate (% mol) |        |           | Heparan sulfate (% mol) |          |          |           |           |          |
|--------|-----------------------------|--------|-----------|-------------------------|----------|----------|-----------|-----------|----------|
|        | Δdi-0S                      | Δdi-4S | Δdi-6S    | Δdi-0S                  | Δdi-NS   | Δdi-6S   | Δdi-S1    | Δdi-S2    | Δdi-triS |
| PlnDI  | 34 (0.88)                   |        | 28 (0.88) | 38 31 (1.15)            | 2 (0.88) | 9 (1.76) | 40 (1.73) | 15 (1.45) | 3 (1.09) |
|        | (1.45)                      |        |           |                         |          |          |           |           |          |

Data are presented as the mean of three independent experiments ± (SEM) restored synergy with VEGF165 in a PlnDI-HS chain dependent manner (Figure 5).

Because the role of HS in heparin-binding growth factor activity may involve interactions between HS, ligand, and cell surface receptors, the ability of PlnDI-HS to modulate VEGF165-induced VEGFR-2 tyrosine phosphorylation was investigated by Western blot using VEGFR-2 (Tyr-951) specific antibodies. VEGFR-2 phosphorylation at Tyr-951 results in recruitment of several adapter proteins whose subsequent downstream signaling supports endothelial cell survival and migration [19]. To perform these studies, we employed bone marrow endothelial cells whose cell surface HS were first removed by exposure to heparinases. Under these conditions, the exogenous addition of PlnDI and VEGF165 (positive control) enhanced VEGFR-2 phosphorylation at Tyr-951 (Figure 6A-B). The signal intensity of phosphorylation increased over time, peaked after ten minutes, then returned to control levels after 20 minutes (Figure 6A-B). The addition of PlnDI, adorned with only HS



chains, enhances Tyr-951 phosphorylation ~3 fold relative to intact PlnDI (Figure 6C). Studies employing PlnDI preparations pre-treated with mixtures of chondroitinase ABC and heparinase enzymes did not completely attenuate phosphorylation (Figure 6C). Heparin

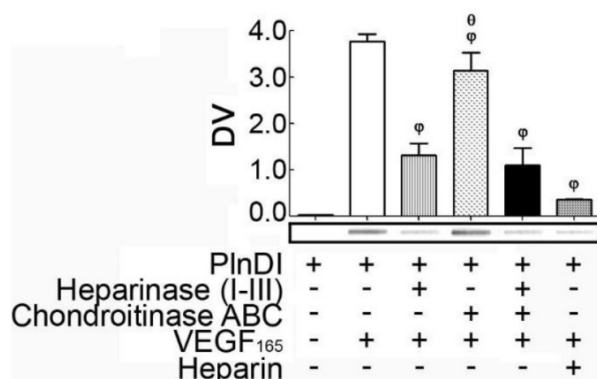


Figure 2 Immunoblot analysis of VEGF165 binding to immobilized PlnDI. PlnDI, undigested or pre-digested with a heparinase cocktail +/- chondroitinase ABC were immobilized on nitrocellulose and incubated with VEGF165. Bound VEGF165 was detected with VEGF specific monoclonal antibodies. Horizontal bars represent the densitometric scanning of slots outlined in rectangular boxes. Data are presented as mean density values (DV) of triplicate determinations  $\pm$  SEM. ( ) different from PlnDI+VEGF165; ( $\theta$ ), different from PlnDI+Hepase+VEGF165.  $p < 0.05$ . addition (positive control) also enhanced VEGFR-2 phosphorylation (Figure 6C).

Relative to either alone, PlnDI/VEGF165 mixtures stimulate peak phosphorylation after only 2.5 minutes (Figure 7A vs. 6A-B). To identify the role of PlnDI-HS in modulating VEGF165 induced VEGFR-2 phosphorylation at Tyr-951, PlnDI preparations adorned with either CS, HS, or without GAGs were pre-mixed with VEGF165. The absence of HS chains on PlnDI reduced the signal intensity of phosphorylation 43% (Figure 7B). In contrast, preparations decorated only with HS chains enhance the signal intensity of phosphorylation ~3 fold (Figure 7B). The absence of CS and HS chains did not completely reduce the intensity of phosphorylation relative to control (VEGF165).

To determine if PlnDI/VEGF165 enhanced VEGFR-2 phosphorylation also promotes downstream signaling, blots were stripped then re-probed with antibodies specific for total and phosphorylated forms of Akt. PlnDI/VEGF165 mixtures enhance the signal intensity of phosphorylated Akt ~4 fold, relative to VEGF165 alone

(Figure 7C), and ~40% of this activity is PlnDI-HS chain dependent. Since PlnDI may modulate phosphorylation via direct interactions with VEGFR-2 or a candidate co-receptor, we performed binding studies with immobilized recombinant VEGFR-2 and NRP-1. PlnDI binds VEGFR-2 and NRP-1 (Figure 8A-B); however, a higher percentage of PlnDI binds NRP-1. The presence of VEGF165 but not VEGF121 (negative control) enhances PlnDI binding to VEGFR-2 (27%) and NRP-1 (13%). The presence of heparin [1 µg/ml] reduces PlnDI binding to NRP-1 more than 60%. In contrast, PlnDI binding to VEGFR-2 was poorly competed away by heparin (Figure 8A-B).

## Discussion

For the first time, we have characterized the ability of recombinant PlnDI to bind VEGF165 and modulate its angiogenic activity, in vitro. We have shown that soluble forms of PlnDI are capable of modulating VEGFR-2 phosphorylation, as well as VEGF165-induced phosphorylation of VEGFR-2, and that the heparan sulfate glyco-saminoglycan chains adorning PlnDI are important for these activities. Together, our observations suggest soluble forms of PlnDI may form and/or stabilize a complex between VEGF165, NRP-1, and VEGFR-2 to enhance angiogenic events and VEGFR-2 signaling in human bone marrow endothelial cells (summarized in Figure 9).

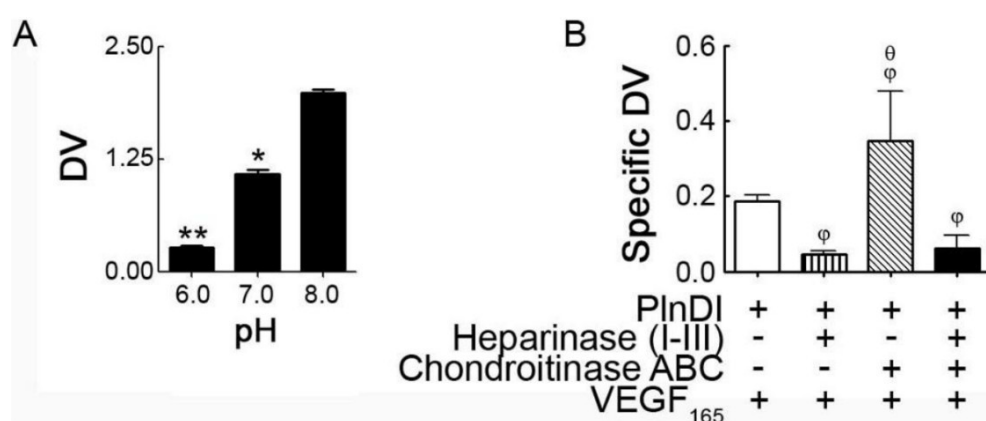


Figure 3 VEGF165 binding to PlnDI in solution is pH dependent. PlnDI pre-incubated with VEGF165 in 25 mM HEPES (pH 6.0, 7.0, or 8.0) was immobilized on nitrocellulose. VEGF165 bound to PlnDI was detected with VEGF specific monoclonal antibodies. (A) pH dependent binding of VEGF165 to PlnDI. (B) The requirement of CS and HS chains for VEGF165 binding to PlnDI (pH 7.0). Data are presented as mean specific



density values (DV) of triplicate determinations  $\pm$  SEM. Specific binding was determined by subtracting the background binding of VEGF165 to nitrocellulose from total bound. (\*) different from pH 8.0; (\*\*) different from pH 7.0; ( ) different from PlnDI+VEGF165; (θ), different from PlnDI+Hepase+VEGF165.  $p < 0.05$ .

In contrast to our previous reports [17,20], the purity of PlnDI employed in the present investigation was enhanced by passage through a Sepharose CL-6B col-umn. SDS-PAGE, Western blot and monosaccharide analysis suggest the molecular weight and GAG chain composition of PlnDI are similar to species previously characterized [20,21]. Moreover, these observations pre-dict our preparation contains at least two species of PlnDI: one adorning predominately CS and the other predominately HS chains. Interestingly, the CS and HS disaccharide composition of PlnDI reported herein is different from species recently characterized by White-lock et al. [22], as well as that reported for full length perlecan purified from bovine rib growth plate cartilage, HUAEC and RT101 cell lines [23-25]. These differences could be due to: 1) cell culture conditions; 2) approaches for purification; and 3) approaches employed for disaccharide analysis. Regardless, since fewer 4-sul-fated CS residues and more 2-sulfated and 6-sulfated HS residues were identified it is reasonable to conclude that the function of PlnDI employed herein is distinct from forms previously reported. Indeed, subtle variations in HS substructure profoundly affect heparin-binding growth factor and receptor interactions, and thus the activity of perlecan [26-28].

While the role(s) of HS chains on perlecan have been most widely investigated with regard to regulation of FGF-2 activity [29,30], few studies have reported on per-lecan-VEGF165 interactions [5,6,22]. Moreover, the GAG modifications required specifically for perlecan-VEGF165 interactions have not been described. Nevertheless, stu-dies with heparin/HS suggest 2-O- and 6-O- sulfation is important for VEGF binding and activity [31-33]. Although the abundance of 2-O- and 6-O-sulfation on

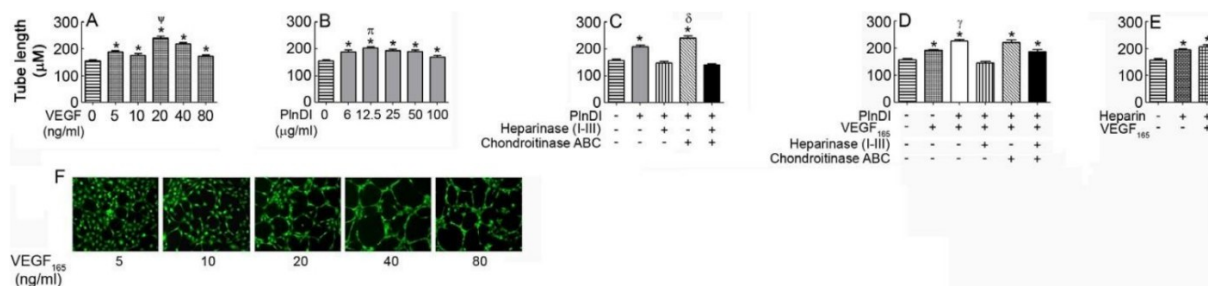
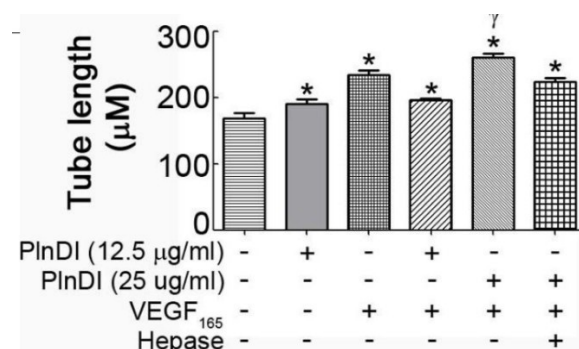


Figure 4 Exogenous PlnDI enhances capillary tube-like formation. Human bone marrow endothelial cells were seeded in 96 well plates pre-coated with growth factor reduced Matrigel and treated with PlnDI pre-incubated +/- VEGF165. Tube length quantification was performed after 18 h on fixed cells, stained with SYTO13. Effect of VEGF165 (A), and PlnDI (B) dose on capillary tube-like formation. (C) PlnDI [12.5 µg/ml] enhanced tube-like formation: Requirement of CS and HS chains. (D) PlnDI/VEGF165 enhanced tube-like formation: Requirement of CS and HS chains on PlnDI. (E) Effect of heparin/VEGF165 mixtures on capillary tube-like formation. (F) Representative images of tube-like formation in panel



PlnDI-HS suggests it harbors the capacity to interact with VEGF165, a correlation between VEGF165 affinity and abundance of a particular disaccharide or the over-all level of HS sulfation has not been observed [31]. Thus, growth factor binding is likely determined by HS domain organization (i.e., length of sulfation and transition domains, as well as their placement along the chain). Since HS chains on recombinant PlnDI are likely to be short (8-10 kDa) relative to those on tumor-derived perlecan (30-70kDa) [21,34,35], we predict 48 residues comprise a single HS chain on PlnDI (based on the molecular weight of repeating units of glucuronic acid and N-acetylglucosamine). Moreover, since six or seven oligosaccharide residues are sufficient to fully occupy the HS binding site for VEGF165 [31], we further predict that six VEGF165 binding sites (maximally) may be available on each HS chain attached to PlnDI.

The HS dependent binding of VEGF165 to immobi-lized PlnDI described herein is consistent with recent reports [5,6]. In contrast, a new communication has reported PlnDI does not bind immobilized VEGF165 [36]. We suspect the concentration and/or the disac-charide composition of PlnDI employed therein may account for the contrasting observations. Our studies with PlnDI in solution suggest VEGF165 binding to PlnDI in solution

is not only HS but pH dependent. The marked reduction in VEGF165 binding to PlnDI under acidic conditions, a novel observation, is consistent with previous publications describing the attenuation of VEGF165 binding with low concentrations of heparin under acidic conditions, and its potentiation at neutral pH [14,37].

To identify the ability of soluble, exogenous PlnDI to modulate VEGF165 activity, in vitro, tube-like formation studies were performed with human bone marrow endothelial cells seeded on growth factor reduced (GFR) Matrigel. We hypothesized that PlnDI/VEGF165 mixtures would enhance the lengths of tube-like structures formed over VEGF165 alone. While our observations support this hypothesis, we were surprised that PlnDI addition, alone, also enhanced the length of tube-like structures. Given our experimental approach, the enhancement of tube-like formation by soluble, exogenous, PlnDI may also reflect interactions with other matrix molecules (i.e., fibronectin and laminin) and heparin-binding growth factors present in GFR Matrigel reported to interact with PlnDI [38]. This possibility, however, should not discount the ability of exogenous PlnDI to interact directly with human bone marrow endothelial cells, or the possibility that the presence of heparin-binding molecules and growth factors may even mask the full activity of PlnDI.

Interestingly, under conditions where bone marrow endothelial cells were pre-treated with a heparinase cocktail, the additive effect of PlnDI/VEGF165 mixtures on tube-like formation was not observed unless the concentration of PlnDI was increased two fold. While these observations suggest PlnDI-HS chains can modulate VEGF165 activity, in vitro, heparin/VEGF165 mixtures (positive control [14,32]), did yield similar results. We remain puzzled by this observation since heparin/ VEGF165 mixtures combine to enhance VEGFR-2 phosphorylation, suggesting heparin is active in our system.

At the cellular/receptor level, we analyzed VEGFR-2 auto-phosphorylation to identify requirements for PlnDI modulation of VEGF165 activity, in vitro. While both VEGFR-1 and VEGFR-2 contribute to VEGF induced signals, VEGFR-2 dominates VEGF induced mitogenic and angiogenic responses in endothelial cells [11,12]. Of the six tyrosine phosphorylation sites identified on the intracellular domain of VEGFR-2, we report on one associated with endothelial cell survival and migration [39]. Together, our observations suggest exogenous soluble PlnDI, alone, can stimulate VEGFR-2 phosphorylation at Tyr-951. Moreover, PlnDI fragments harboring only HS chains further enhance VEGFR-2 phosphorylation, suggesting the presence of CS chains masks activity. These studies importantly extend those recently reported for full length perlecan [6] by demonstrating

delivery of PlnDI or co-delivery with VEGF165 are sufficient to enhance VEGFR-2 phosphorylation, and promote downstream signaling (i.e., increased Akt phosphorylation). Given our approach (i.e., the use of cells in suspension), our observations suggest PlnDI/VEGF

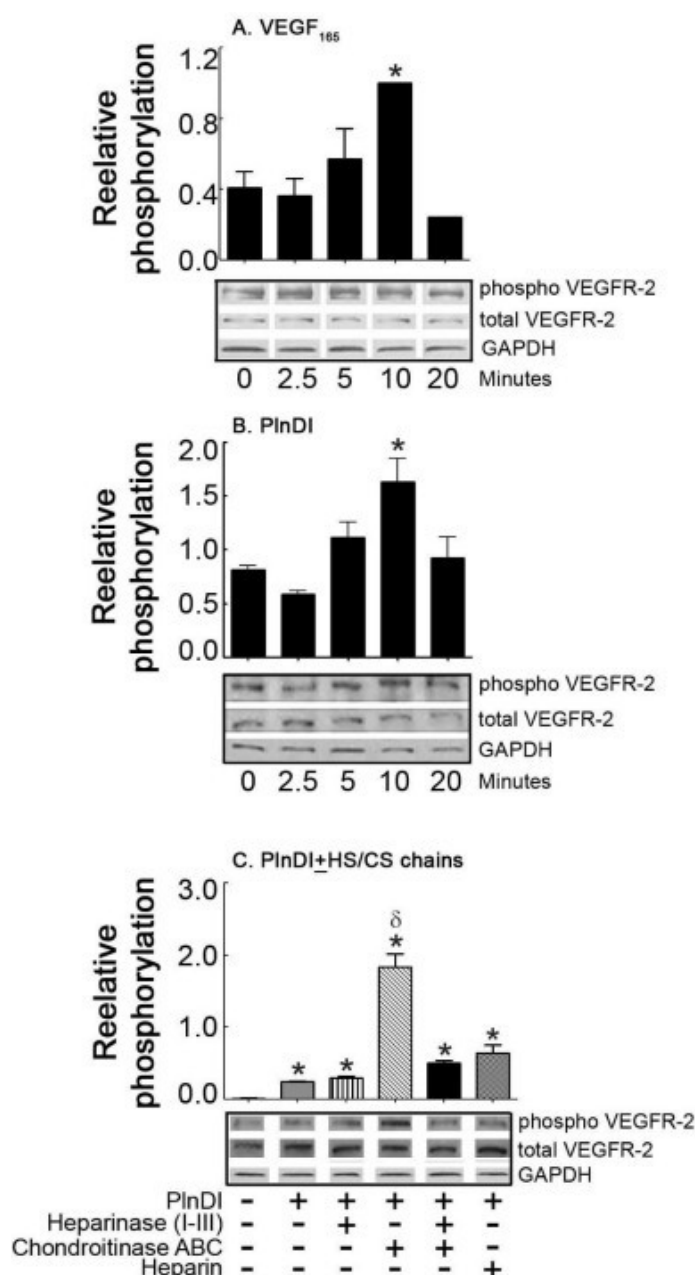


Figure 6 PlnDI stimulates VEGFR-2 (Tyr-951) phosphorylation. Human bone marrow endothelial cells without cell surface HS were incubated with VEGF165 or PlnDI for 0, 2, 5, 10 and 20 minutes. Cell lysates were analyzed for VEGFR-2 phosphorylation by Western blot using anti-phospho and total VEGFR-2 tyrosine residue 951 specific antibodies. Time dependant increase in VEGFR-2 phosphorylation induced by: (A) VEGF165 and (B) PlnDI. (C) PlnDI induced VEGFR-2 phosphorylation: Requirement for CS and HS chains. Exogenous heparin served as a positive control and GAPDH as loading control. Horizontal bars represent the densitometric scanning of bands outlined in rectangular boxes. Data are presented as the mean density values (DV) from triplicate determinations  $\pm$  SEM. (\*) different from 0 min or media. ( $\delta$ ) different from PlnDI.  $p < 0.05$

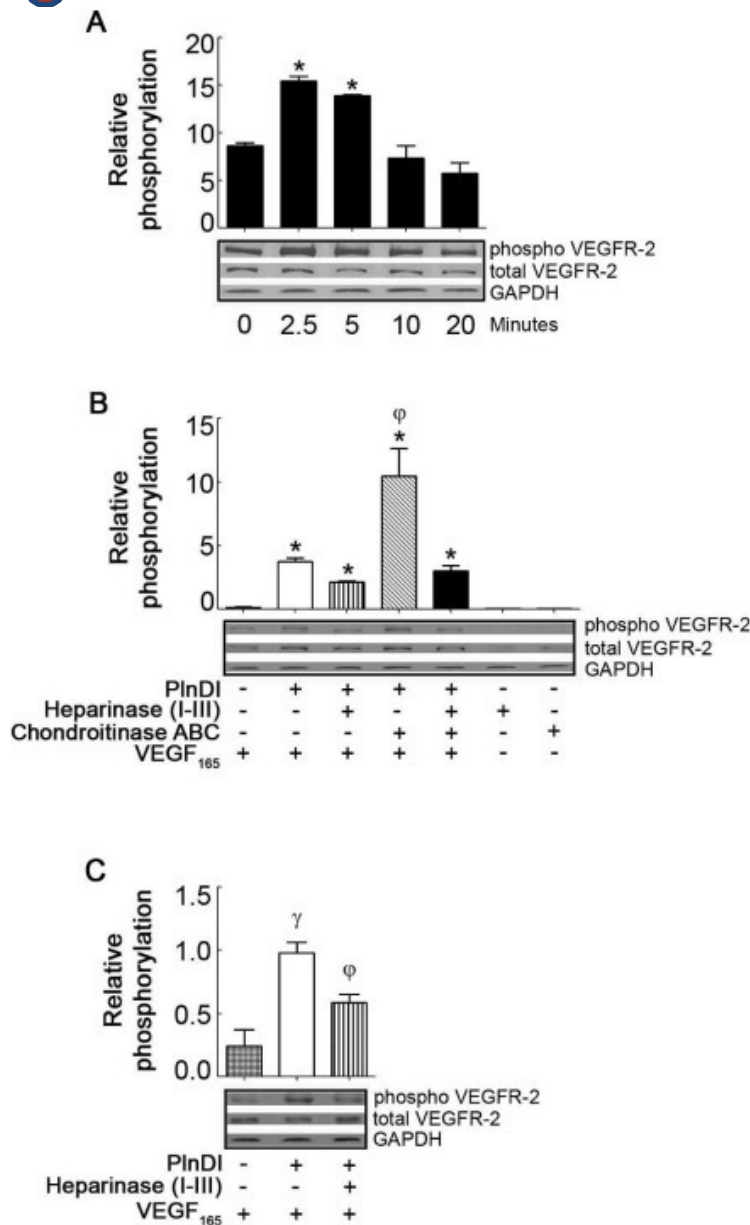
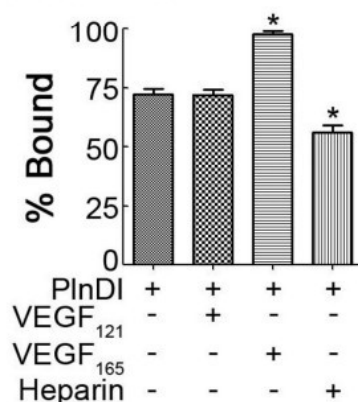


Figure 7 PInDI/VEGF<sub>165</sub> mixtures enhance VEGFR-2 (Tyr-951) phosphorylation. Human bone marrow endothelial cells without cell surface HS were incubated with PInDI/VEGF<sub>165</sub> mixtures for either 0, 2, 5, 10, or 20 min. Cell lysates were analyzed for VEGFR-2 (A and B) or Akt (C) phosphorylation by Western blot using anti-phospho and total VEGFR-2 (tyrosine residue 951) and Akt specific antibodies. (B) PInDI/VEGF<sub>165</sub>



enhanced VEGFR-2 phosphorylation (at min 2.5): Requirement of CS and HS chains on PlnDI. Horizontal bars represent the densitometric scanning of bands outlined in rectangular boxes. GAPDH was assessed as a loading control. Data are presented as the mean of three independent experiments  $\pm$  SEM. (\*) different from 0 min; (g) different from VEGF165; ( ) different from PlnDI+VEGF165.  $p < 0.05$ .

## A. VEGFR-2



## B. NRP-1

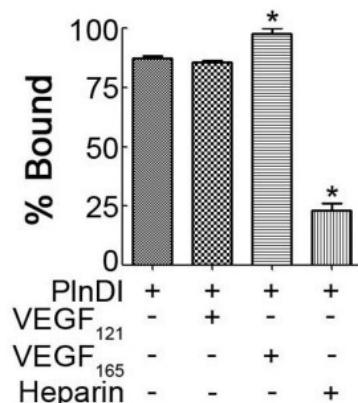


Figure 8 PlnDI binds NRP-1 and VEGFR-2. Recombinant NRP-1 and VEGFR-2 coated plates were incubated with PlnDI +/- VEGF165 or VEGF121. PlnDI binding was detected and quantified using an ELISA based approach that employed anti-PlnDI specific antibodies. For competitive inhibition assays, PlnDI was pre-incubated with heparin [1  $\mu$ g/ml]. (A) PlnDI binding to VEGFR-2. (B) PlnDI binding to NRP-1. Data are presented as a percentage of total bound. (\*) different from PlnDI.  $p < 0.05$  mixtures enhance survival signaling (increased Akt phosphorylation) of human bone marrow endothelial cells, in vitro. Consistent with this

conclusion, our unpublished observations suggest VEGFR-2 phosphorylation at Tyr-1175 and Tyr 1214, and phosphorylation of p38 MAPK, Erk1/2 (events associated with endothelial cell proliferative and migratory states) [39], are unaltered.

Finally, to determine if PlnDI has the capacity to bind and modulate the activity of VEGFR-2 directly, we performed PlnDI binding studies against immobilized VEGFR-2, and NRP-1. Outcomes from these studies suggest PlnDI-HS chains, similar to heparin/HS, harbor the capacity to interact with VEGFRs and co-receptors [15,32,40], and enhance VEGFR-2 signaling [41]. We suspect PlnDI-HS chain binding to NRP-1 occurs via its heparin binding domain [15]. In contrast, PlnDI binding to VEGFR-2 is less dependent on HS chains. Heparin concentrations up to [100 µg/ml] did not appreciably alter binding (unpublished observations). Interestingly, the pre-sence of VEGF165 enhances PlnDI binding to VEGFR-2, suggesting the formation of a complex between PlnDI/ VEGF165/VEGFR-2 is possible. Our observations also suggest that modulation of VEGFR-2 signaling by PlnDI may involve complex interactions with more than one ligand.

### **Conclusion**

The findings presented herein demonstrate exogenous, soluble, recombinant PlnDI is sufficient to bind and modulate the activity of the VEGFR-2 signaling complex via HS interactions, in vitro. Moreover, PlnDI may have activities independent of those with heparin-binding growth factors in supporting tube-like formation, in vitro. Figure 9 provides a simplified visual depiction of how PlnDI may impact angiogenic events in the absence or presence of VEGF165. PlnDI unbound or bound to VEGF165 is liberated via cleavage within its SEA module [42] or the single immunoglobulin G-like region of domain II [43,44] during matrix turnover, wound healing, or disease progression. In the absence of VEGF165, PlnDI-HS may bind to NRP-1, VEGFR-2, or support complex formation with both to signal downstream angiogenic events. When VEGF165 is present PlnDI interactions with NRP-1 and VEGFR-2 are optimized, leading to enhanced downstream signaling and angiogenesis.

### **Methods**

#### **Materials**

Recombinant human VEGF165, VEGFR-2, NRP-1, and anti-VEGF165 monoclonal antibodies were procured from R&D systems, Inc. (Minneapolis, MN). Growth factor reduced Matrigel was purchased from BD Bios-

ciences (San Jose, CA). Goat polyclonal antibodies to GAPDH were purchased from Genscript (Piscataway,

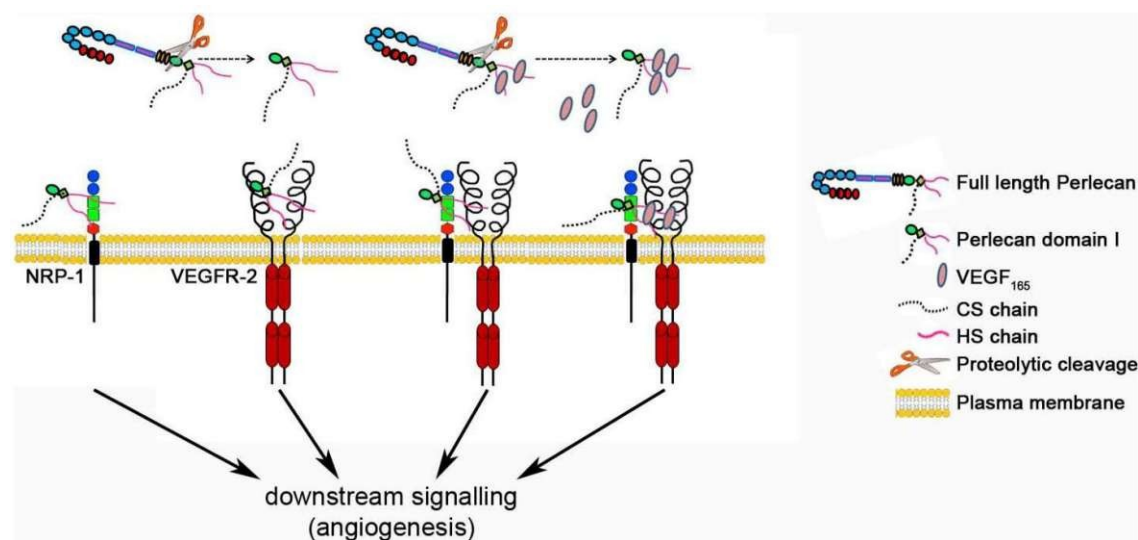


Figure 9 Model: PlnDI interactions with the VEGFR-2 signaling complex. N-terminal domain I of perlecan, unbound or bound to VEGF165, liberated by proteolytic cleavage (scissors) during extracellular matrix turnover stimulates angiogenesis by direct interactions with: 1) NRP-1; 2) VEGFR-2; and 3) NRP-1 and VEGFR-2. PlnDI may enhance VEGF165 stimulated pro-angiogenic events by stabilizing NRP-1/VEGF165/VEGFR-2 interactions. NJ). Rabbit polyclonal antibodies for phospho- and total- VEGFR-2, and Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling (Danvers, MA), respectively. Anti-Perlecan domain I monoclonal antibodies (CSI 001-71) were purchased from the Antibody Shop (Denmark). Anti-Perlecan domain IV antibodies were purchased from Millipore (Temecula, CA). Heparin, heparinase I, II and III and protease free chondroitinase ABC were purchased from Sigma (St. Louis, MO). Heparitinase II enzyme, 3G10 antibodies, and unsaturated heparan/heparin-disaccharide standards were purchased from Seikagaku Corp (Japan).

## Cell Culture

Human bone marrow endothelial cells, provided by Dr. G Almeida-Porada (University of Nevada, Reno, [45]), were cultured in M199 media supplemented with endothelial cell growth supplement (R&D systems Inc, Minneapolis, MN), 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin, 2 mM glutamax and heparin (15 U/ml). Cells were sub-cultured when 80-90% confluent using 0.05% (v/v) trypsin/EDTA. All cul-

tures were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

Sepharose CL-6B enrichment of Recombinant PlnDI Recombinant perlecan domain I (PlnDI) was prepared as described previously [17]. PlnDI was enriched by passage through a Sepharose CL-6B column (1 × 50 cm), pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.6 containing 6 M guanidine-HCl and 0.5 M NaCl. Fractions were assayed for uronic acid by carbazole method [46], and protein by micro BCA assay (Pierce, Rockford, IL). PlnDI purity was assessed by SDS-PAGE (i.e., Alcian blue and Coomassie blue staining) and Western blotting (see below).

### **Western Blotting**

PlnDI (25 µg), untreated or pre-digested with heparinase cocktail (mixture of heparinases I, II and III, 2.5 Sigma units each) and/or chondroitinase ABC, were electro-phoresed on 3-8% Tris-acetate gels (Invitrogen, CA), then transferred to nitrocellulose. Membranes were probed with anti-PlnDI monoclonal antibodies diluted (1:200) in phosphate buffered saline (PBS) with 0.1% (v/v) Tween-20 (PBST), containing 3% (w/v) BSA. Primary antibodies were detected with anti-mouse IgG secondary antibodies conjugated to peroxidase and visualized by incubation with enhanced chemiluminescence reagent (ECL, GE Healthcare), and exposure to film.

### **Chondroitinase ABC and Heparinase digestion**

For chondroitinase ABC digestion PlnDI (25 µg) was incubated with chondroitinase ABC (20 mU) in 25 µl of 100 mM/L Tris-HCl, pH 8.0, containing 30 mM/L sodium acetate and 0.01% (w/v) BSA at 37°C for 5 hours. For heparinase digestion, PlnDI was incubated with a heparinase cocktail in 25 µl of PBS containing 4 mM CaCl<sub>2</sub> and protease inhibitors for 12 hours at room temperature.

### **Immunoassays**

Solid phase binding assays were performed as described previously [17]. For solution phase binding assays, PlnDI (5 µg) untreated, or pre-digested with a heparinase cocktail and/or chondroitinase ABC was pre-incubated with 20 ng of VEGF165 in PBS containing 3% (w/v) BSA, or 25 mM HEPES at either pH 8.0, 7.0, or 6.0 [37], or 50 mM Tris-HCl (pH 8.0), PBS (pH 7.0), 50 mM sodium acetate (pH 6.0) for 1 hr at room temperature. Samples were subsequently blotted onto nitrocellulose, and blocked. Bound VEGF165 was detected with anti-VEGF165 antibodies (1 µg/ml in 3% (w/v) BSA in PBST). Primary antibodies were detected

with anti-mouse IgG secondary antibodies conjugated to HRP and visualized as described for Western blotting. Binding was quantified by densitometry and expressed as mean density values (DV) from triplicate assays. Specific binding was determined by subtracting VEGF165 background from total bound [18].

### **Capillary Tube-like Assay**

Growth factor reduced (GFR) Matrigel was added to wells of ice-cold 96-well plates (70 µl/well) for 6 seconds. Excess was removed, leaving a thin coating. Plates were incubated for 6 minutes on ice, 20 minutes at room temperature, and finally warmed for 20 minutes at 37°C. Bone marrow endothelial cells were seeded (6,500 cells/well) in serum free RPMI 1640 media containing 1% (w/v) penicillin/streptavidin, 2 mM glutamax without growth supplements. After cell attachment, the media was replaced with media containing one or more supplements [i.e., PlnDI (12.5 µg/ml), untreated or pre-digested with a heparinase cocktail and/or chondroitinase ABC, heparin (4.0 µg/ml), VEGF165 (20 ng/ml)]. For assays conducted in the absence of cell surface heparin sulfate, human bone marrow endothelial cells were cultured for 15 minutes under serum free conditions in RPMI 1640 media supplemented with heparinase cocktail [32]. Such treatments temporarily remove more than 95% of cell surface HS. Prior to seeding cells were washed twice with RPMI 1640 media. To quantify tube-like formation cells were fixed (4% (v/v) paraformaldehyde) after 18 h, stained (SYTO13, Invitrogen, CA), then photographed with a SPOT CCD camera affixed to an inverted microscope equipped for epifluorescence. Nine random fields, representing 80% of each well, were analyzed for three angiogenic parameters: average tube length (defined as three or more cells connected lengthwise, and exceeding 100 µm in length; [47], number of tube-like structures, and the number of branch points, using Image J software (NIH). When several tube-like structures merged together or branched, the total length was calculated as the sum of the individual branches. All tube-like formation studies were conducted in quadruplicate wells, and repeated at least three times. Since the outcomes of each angiogenic parameter were similar only average tube length is reported. Note: All supplement concentrations employed herein are optimal, and were determined empirically over a broad range. As a control for enzyme activity, assays were also conducted with supplements containing heat inactivated chondroitinase ABC and/or heparinase cocktail.

### **Receptor Phosphorylation studies**

Bone marrow endothelial cells, cultured to 80% confluence, were rinsed twice with serum free media, and then

cultured for 24 hrs under serum free conditions. Cells were pre-incubated with a heparinase cocktail to remove cell surface heparan sulfate (as described above) then incubated with serum free media containing supplements (PlnDI, VEGFs, and heparin, as described above) for either 0, 2.5, 5, 10 or 20 minutes. After washing (ice-cold PBS), the cells were lysed [10 mM Tris-HCl, buffer pH 7.4 containing 140 mM NaCl, 0.2% (v/v) Triton X-100, 1.5 mM EDTA, 1 mM Na<sub>3</sub>PO<sub>4</sub>, 25 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>, protease inhibitors (Roche Diagnostics, Lewes, UK)], and total protein concentrations determined (micro BCA assay). For Western blotting, 30 µg of each sample was loaded onto 7% Tris-acetate gels, electrophoresed under reducing conditions, then transferred onto nitrocellulose. Membranes were probed with anti-phospho-VEGFR-2 (tyrosine residue (Tyr)-951), phospho-Akt, anti total-VEGFR-2, total-Akt, and anti-GAPDH antibodies. Primary antibodies were detected and visualized as described for Western blotting.

## **Enzyme Linked Immunosorbant Assay**

Recombinant proteins (NRP-1 and VEGFR-2) were allowed to bind overnight (4°C) in 96-well plates (100 ng/well; Maxi Sorp, Nunc). After several washes and blocking with PBS containing 3% BSA, PlnDI (5 µg/ well) with or without VEGF121, VEGF165, or heparin [0.01-1000 µg/mL] was added. After 2 h, and several washes with PBS containing 0.05% Tween 20, the plates were incubated with anti-PlnDI antibodies (1:500 dilution) for 1 h. Primary antibodies were detected with anti-rabbit IgG secondary antibodies conjugated to HRP (1:8000). Each well was subsequently incubated with tet-ramethylbenzidine (KPL; 100 µl/well) for 10 min under gentle agitation. Color development was stopped with 50 µL of 0.5 N H<sub>2</sub>SO<sub>4</sub>. Binding was quantified by measuring absorbance at 450 nm. Unless indicated, all incubations were performed at room temperature.

## **Monosaccharide analysis**

As done previously [46], PlnDI (20 µg) was hydrolyzed with 4 M HCl at 100°C for 6 h, then dried in a Speed-Vac. Residues were dissolved in HPLC grade water then analyzed on a CarboPac PA1 high pH anion-exchange column (4 × 250 mm) using Dionex BioLC HPLC coupled to a pulse amperometric detector.

## **Disaccharide composition analysis of GAG chain**

As done previously [48], CS disaccharides, released from PlnDI (20 µg) following digestion with chondroitinase ABC [(20 mU) in 50 µl of 100 mM Tris-HCl, 30 mM NaOAc, pH 8.0, containing 0.01% (w/v)



BSA at 37°C for 5 h] were analyzed by HPLC on the 4.6 × 250 mm amine-bonded silica PA03 column. Unsaturated HS dis-accharides released from PlnDI following digestion with heparinase cocktail were analyzed as above. Commercially prepared bovine tracheal CS disaccharide standards ( $\Delta$ Di-0 S,  $\Delta$ Di-4 S and  $\Delta$ Di-6S), and unsaturated heparan/heparin-disaccharide standards were used to determine standard migration positions and for quantitation.

### **Statistical analysis**

All experiments were conducted in triplicate, repeated at least three times, and analyzed by two-tailed paired Student's t-test using GraphPad Prism version 5.0 for Windows (San Diego California USA). Differences were considered significant at  $P < 0.05$ . All results are pre-sented as means  $\pm$  standard error of the mean.

### **Abbreviations**

**CS:** chondroitin sulfate; **GAG:** glycosaminoglycan; **HSPG:** heparan sulfate proteoglycan; **HS:** heparan sulfate; **NRP-1:** Neuropilin 1; **PlnDI:** recombinant perlecan domain I; **VEGF:** vascular endothelial growth factor; **VEGFR-2:** vascular endothelial growth factor receptor-2; **p-VEGFR-2:** phosphor-VEGFR-2; **t-VEGFR-2:** total-VEGFR-2.

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## **Authors' contributions**

AM performed all experiments described herein. RRG conceived the study, and participated in its design and coordination. All the authors contributed equally to data analysis, interpretation, and communication of the findings. All authors have read and approved the final version of the manuscript.

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