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

Arivalagan Muthusamy<sup>1</sup>, Carlton R Cooper<sup>2</sup>, Ronald R Gomes Jr<sup>1\*</sup>

## 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 (VEGF<sub>165</sub>) enhanced capillary tube-like formation, and VEGF receptor-2 phosphorylation of human bone marrow endothelial cells, *in vitro*.

**Results:** In solution, PlnDI binds VEGF<sub>165</sub> in a heparan sulfate and pH dependent manner. Capillary tube-like formation is enhanced by exogenous PlnDI; however, PlnDI/VEGF<sub>165</sub> mixtures combine to enhance formation beyond that stimulated by either PlnDI or VEGF<sub>165</sub> alone. PlnDI also stimulates VEGF receptor-2 phosphorylation, and mixtures of PlnDI/VEGF<sub>165</sub> 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 VEGF<sub>165</sub> but not VEGF<sub>121</sub> 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 VEGF<sub>165</sub> 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 VEGF<sub>165</sub> to coordinate developmental angiogenesis by modulating VEGF<sub>165</sub>/VEGFR-2 signaling [5,6]. However, a role for soluble forms of PlnDI and the mechanism(s) by which it modulates VEGF<sub>165</sub>/VEGFR-2 signaling is unclear.

\* Correspondence: rgomes@hmc.psu.edu

<sup>1</sup>Department of Orthopaedics and Rehabilitation, Penn State College of Medicine, Hershey, Pennsylvania 17033, USA

Full list of author information is available at the end of the article



(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 PInDI was confirmed by Western blotting using anti-PInDI specific antibodies (CSI-0071) and antibodies (3G10) to anti- $\Delta$ -heparan sulfate that recognize heparan sulfate neo-epitopes, generated following heparinase cleavage (arrow Figure 1C and 1D). Neither antibody recognized undigested products; however, anti-PInDI 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 PInDI suggests a protein and uronic acid content of 49% and 37%, respectively (Table 1). Hexosamine (monosaccharide) compositional analysis revealed PInDI GAGs are composed predominantly of galactosamine (60%) relative to glucosamine (40%) (Table 1). The disaccharide composition of purified PInDI revealed 6-sulfated disaccharide as the major  $\Delta$ di-CS with lesser amounts of nonsulfated and 4-sulfated disaccharides (Table 2). The major  $\Delta$ di-HS derived from PInDI was nonsulfated and  $\Delta$ di-S<sub>1</sub> with considerable, but lesser amounts of  $\Delta$ di-S<sub>2</sub>, -6-sulfated, -N-sulfated, and -triS disaccharides (Table 2). The HS GAG chains on PInDI contain approximately 3 fold more 6-O- than 2-O-sulfation.

#### VEGF<sub>165</sub> binds to PInDI in a heparan sulfate dependent manner

To identify requirement(s) for VEGF<sub>165</sub> binding to PInDI, both solid and solution phase binding assays were performed. In solid phase binding assays, immobilized PInDI binds VEGF<sub>165</sub> in a heparan sulfate dependent manner (Figure 2). Heparinase cocktail treatment of PInDI, prior to immobilization on nitrocellulose, reduced VEGF<sub>165</sub> binding by ~75% (Figure 2). In contrast, pre-digestion with chondroitinase ABC did not alter VEGF<sub>165</sub> binding. Studies with the PInDI protein core, prepared following digestion with a mixture of both enzymes, suggest VEGF<sub>165</sub> poorly binds this region. VEGF antibodies do not bind immobilized PInDI (Figure

2). In competitive inhibition assays, heparin [0.25  $\mu$ g/ml] prevented ~80% of VEGF<sub>165</sub> binding to PInDI (Figure 2).

In solution, requirements for VEGF<sub>165</sub> binding to PInDI 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, VEGF<sub>165</sub> binding was reduced by 50% and 80%, respectively (Figure 3A). To identify VEGF<sub>165</sub> specific binding, the background binding of VEGF<sub>165</sub> to nitrocellulose was subtracted from total bound to PInDI [18]. Employing this approach, PInDI-HS chains account for nearly all VEGF<sub>165</sub> binding, and the presence of CS chains masks VEGF<sub>165</sub> interaction with HS (Figure 3B). In panel B, neutral pH was chosen to more closely reflect tissue culture conditions of subsequent experiments.

#### PInDI modulation of VEGF<sub>165</sub> bio-activity

To identify a role for PInDI in modulating VEGF<sub>165</sub> activity *in vitro*, human bone marrow endothelial cells were employed in two independent assays: 1) VEGF<sub>165</sub>-enhanced capillary tube-like formation; 2) VEGF<sub>165</sub>-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 VEGF<sub>165</sub> +/- PInDI was quantified. Under serum free conditions, the addition of soluble VEGF<sub>165</sub> (positive control) and PInDI demonstrated dose dependent increases in lengths of tube-like structures formed (Figure 4A-B and 1F). Optimal concentrations for VEGF<sub>165</sub> [20 ng/ml] and PInDI [12.5  $\mu$ g/ml] increased tube-like formation 35% and 24%, respectively.

Studies employing PInDI, pre-treated with either chondroitinase ABC and/or a heparinase cocktail suggests the ability of PInDI to enhance tube-like formation is HS chain dependent (Figure 4C). Moreover, PInDI activity is further enhanced when its CS chains are removed. Interestingly, PInDI/VEGF<sub>165</sub> mixtures combine to enhance tube-like formation 16% relative to VEGF<sub>165</sub> alone (Figure 4D). The synergy between PInDI and VEGF<sub>165</sub> is PInDI-HS chain dependent (Figure 4D). PInDI protein core/VEGF<sub>165</sub> mixtures produce tube-like structures indifferent from those by VEGF<sub>165</sub> alone. Unexpectedly, heparin/VEGF<sub>165</sub> 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, VEGF<sub>165</sub> and PInDI enhance tube-like formation (Figure 5); however, PInDI/VEGF<sub>165</sub> mixtures did not combine to further enhance the lengths of tube-like structures. Subsequent dose response studies suggested elevated concentrations of VEGF<sub>165</sub> and PInDI are required for maximal activity. Increasing PInDI concentration two fold [25.0  $\mu$ g/ml]

**Table 1 Biochemical composition of PInDI**

Sample	Composition (% dry weight)		Hexosamine (% mol)	
	Protein	Uronic acid	GalN	GlcN
PInDI	49 (0.37)	37 (0.29)	60 (0.87)	40 (0.88)

Data are presented as the mean of three independent experiments  $\pm$  (SEM).

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

Sample	Chondroitin sulfate (% mol)				Heparan sulfate (% mol)				
	$\Delta$ di-0S	$\Delta$ di-4S	$\Delta$ di-6S	$\Delta$ di-0S	$\Delta$ di-NS	$\Delta$ di-6S	$\Delta$ di-S <sub>1</sub>	$\Delta$ di-S <sub>2</sub>	$\Delta$ di-triS
PlnDI	34 (0.88)	28 (0.88)	38 (1.45)	31 (1.15)	2 (0.88)	9 (1.76)	40 (1.73)	15 (1.45)	3 (1.09)

Data are presented as the mean of three independent experiments  $\pm$  (SEM).

restored synergy with VEGF<sub>165</sub> in a PlnDI-HS chain dependent manner (Figure 5).

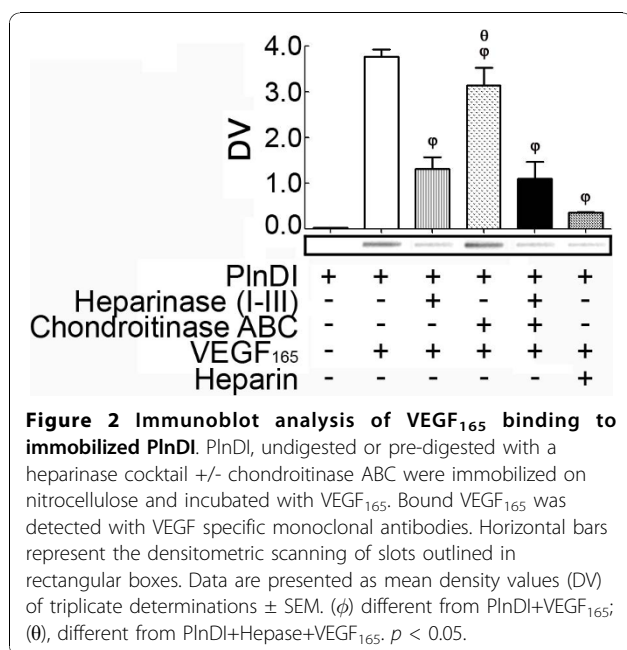
Because the role of HS in heparin-binding growth factor activity may involve interactions between HS, ligand, and cell surface receptors, the ability of PlnDI-HS to modulate VEGF<sub>165</sub>-induced VEGFR-2 tyrosine phosphorylation was investigated by Western blot using VEGFR-2 (Tyr-951) specific antibodies. 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 VEGF<sub>165</sub> (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  $\sim$ 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

addition (positive control) also enhanced VEGFR-2 phosphorylation (Figure 6C).

Relative to either alone, PlnDI/VEGF<sub>165</sub> mixtures stimulate peak phosphorylation after only 2.5 minutes (Figure 7A vs. 6A-B). To identify the role of PlnDI-HS in modulating VEGF<sub>165</sub> induced VEGFR-2 phosphorylation at Tyr-951, PlnDI preparations adorned with either CS, HS, or without GAGs were pre-mixed with VEGF<sub>165</sub>. 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  $\sim$ 3 fold (Figure 7B). The absence of CS and HS chains did not completely reduce the intensity of phosphorylation relative to control (VEGF<sub>165</sub>).

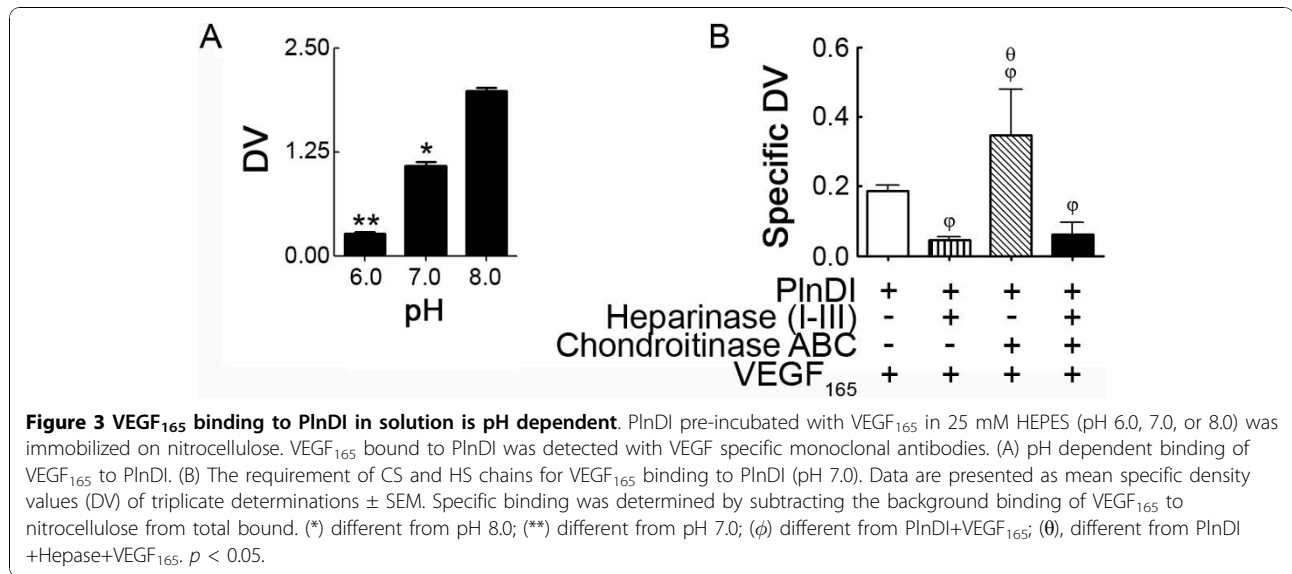
To determine if PlnDI/VEGF<sub>165</sub> enhanced VEGFR-2 phosphorylation also promotes downstream signaling, blots were stripped then re-probed with antibodies specific for total and phosphorylated forms of Akt. PlnDI/VEGF<sub>165</sub> mixtures enhance the signal intensity of phosphorylated Akt  $\sim$ 4 fold, relative to VEGF<sub>165</sub> alone (Figure 7C), and  $\sim$ 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 VEGF<sub>165</sub> but not VEGF<sub>121</sub> (negative control) enhances PlnDI binding to VEGFR-2 (27%) and NRP-1 (13%). The presence of heparin [1  $\mu$ g/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

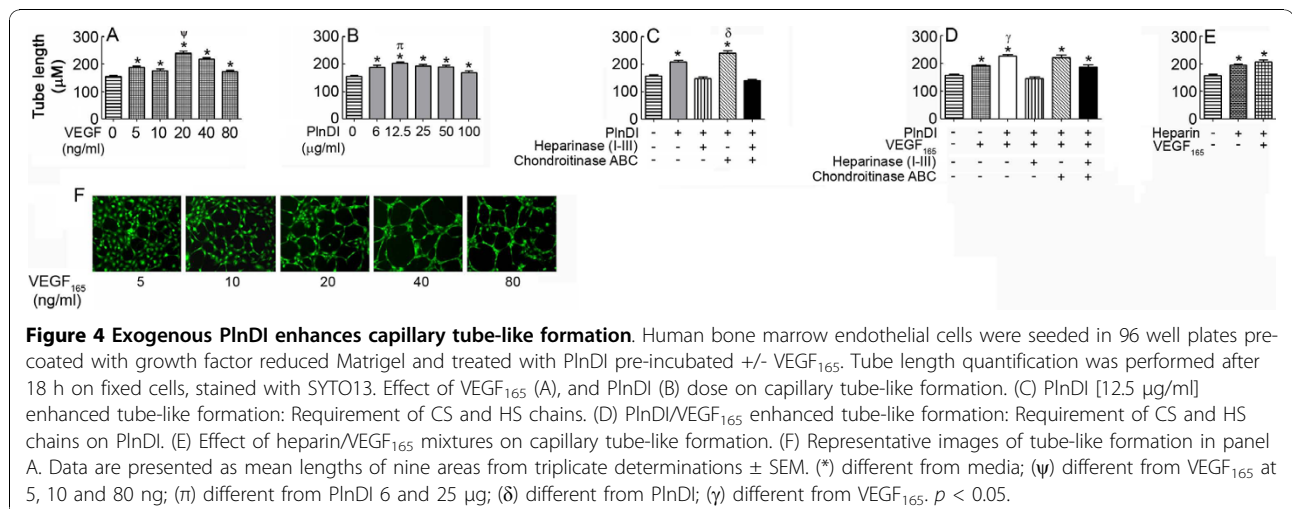
For the first time, we have characterized the ability of recombinant PlnDI to bind VEGF<sub>165</sub> and modulate its angiogenic activity, *in vitro*. We have shown that soluble forms of PlnDI are capable of modulating VEGFR-2 phosphorylation, as well as VEGF<sub>165</sub>-induced phosphorylation of VEGFR-2, and that the heparan sulfate glycosaminoglycan chains adorning PlnDI are important for these activities. Together, our observations suggest soluble forms of PlnDI may form and/or stabilize a complex between VEGF<sub>165</sub>, NRP-1, and VEGFR-2 to enhance angiogenic events and VEGFR-2 signaling in human bone marrow endothelial cells (summarized in Figure 9).



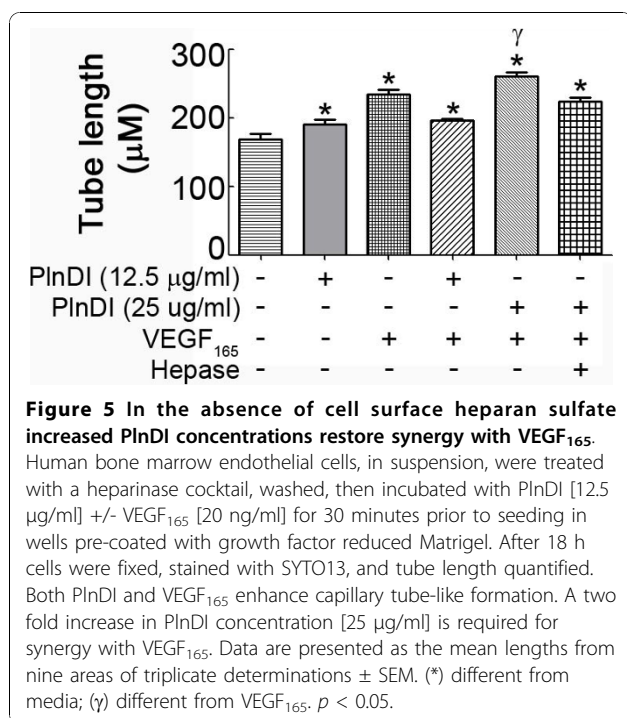
In contrast to our previous reports [17,20], the purity of PlnDI employed in the present investigation was enhanced by passage through a Sepharose CL-6B column. SDS-PAGE, Western blot and monosaccharide analysis suggest the molecular weight and GAG chain composition of PlnDI are similar to species previously characterized [20,21]. Moreover, these observations predict 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-sulfated 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 perlecan-VEGF<sub>165</sub> interactions [5,6,22]. Moreover, the GAG modifications required specifically for perlecan-VEGF<sub>165</sub> interactions have not been described. Nevertheless, studies 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







PlnDI-HS suggests it harbors the capacity to interact with VEGF<sub>165</sub>, a correlation between VEGF<sub>165</sub> affinity and abundance of a particular disaccharide or the overall 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 VEGF<sub>165</sub> [31], we further predict that six VEGF<sub>165</sub> binding sites (maximally) may be available on each HS chain attached to PlnDI.

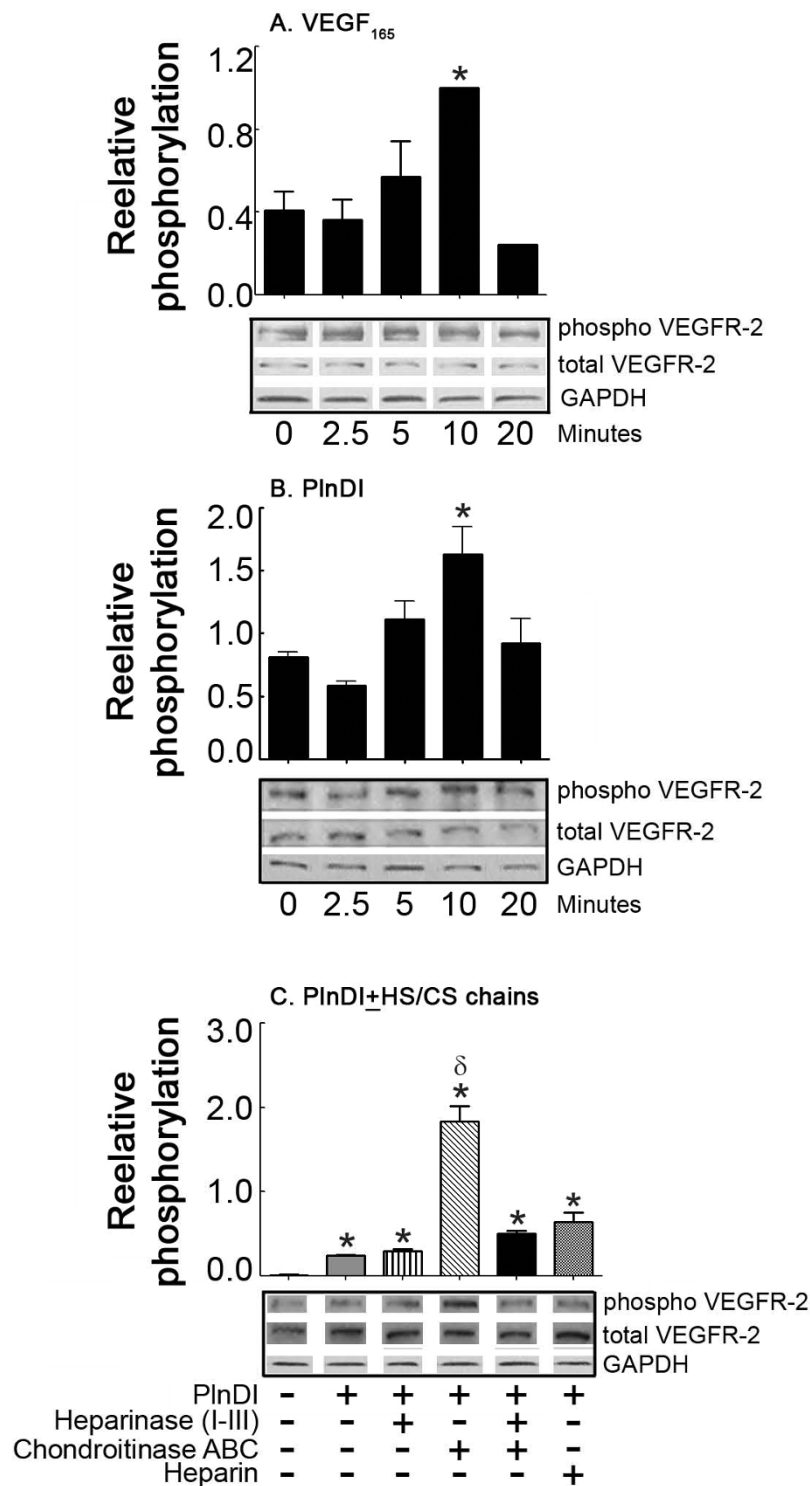
The HS dependent binding of VEGF<sub>165</sub> to immobilized PlnDI described herein is consistent with recent reports [5,6]. In contrast, a new communication has reported PlnDI does not bind immobilized VEGF<sub>165</sub> [36]. We suspect the concentration and/or the disaccharide composition of PlnDI employed therein may account for the contrasting observations. Our studies with PlnDI in solution suggest VEGF<sub>165</sub> binding to PlnDI in solution is not only HS but pH dependent. The marked reduction in VEGF<sub>165</sub> binding to PlnDI under acidic conditions, a novel observation, is consistent with previous publications describing the attenuation of VEGF<sub>165</sub> binding with low concentrations of

heparin under acidic conditions, and its potentiation at neutral pH [14,37].

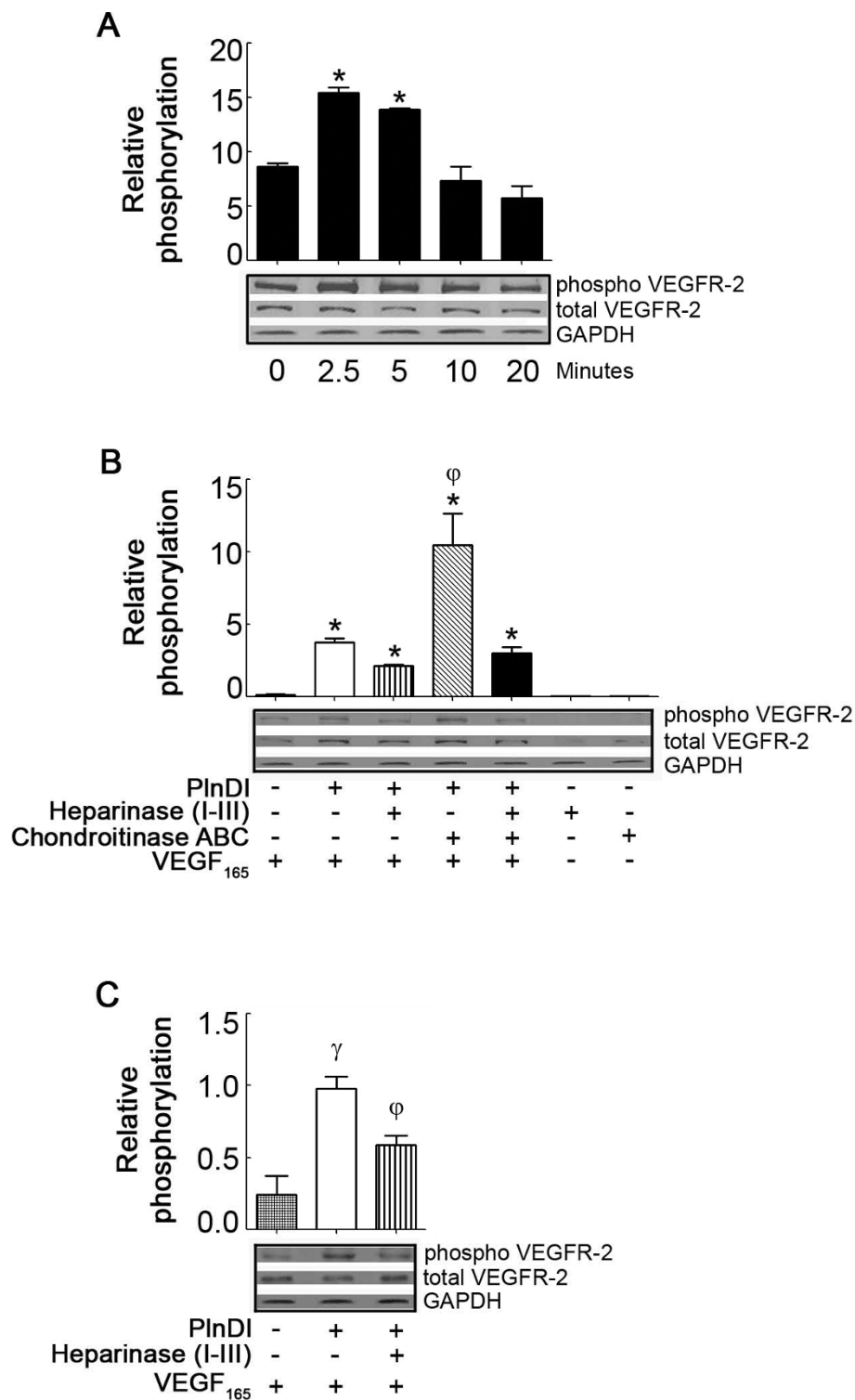
To identify the ability of soluble, exogenous PlnDI to modulate VEGF<sub>165</sub> activity, *in vitro*, tube-like formation studies were performed with human bone marrow endothelial cells seeded on growth factor reduced (GFR) Matrigel. We hypothesized that PlnDI/VEGF<sub>165</sub> mixtures would enhance the lengths of tube-like structures formed over VEGF<sub>165</sub> 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/VEGF<sub>165</sub> 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 VEGF<sub>165</sub> activity, *in vitro*, heparin/VEGF<sub>165</sub> mixtures (positive control [14,32]), did yield similar results. We remain puzzled by this observation since heparin/VEGF<sub>165</sub> 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 VEGF<sub>165</sub> 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 VEGF<sub>165</sub> 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<sub>165</sub>

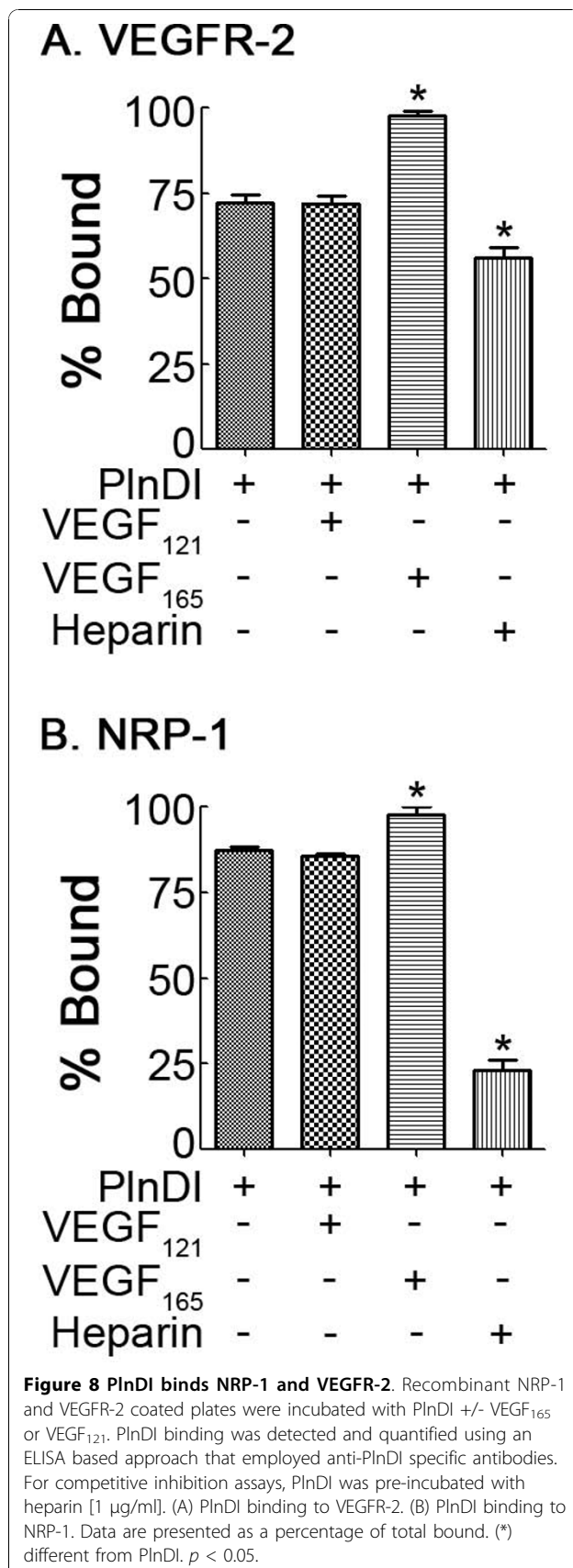


**Figure 6 PlnDI stimulates VEGFR-2 (Tyr-951) phosphorylation.** Human bone marrow endothelial cells without cell surface HS were incubated with VEGF<sub>165</sub> or PlnDI for 0, 2, 5, 10 and 20 minutes. Cell lysates were analyzed for VEGFR-2 phosphorylation by Western blot using anti-phospho and total VEGFR-2 tyrosine residue 951 specific antibodies. Time dependant increase in VEGFR-2 phosphorylation induced by: (A) VEGF<sub>165</sub> 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 ± SEM. (\*) different from 0 min or media. (δ) different from PlnDI.  $p < 0.05$ .



**Figure 7 PlnDI/VEGF<sub>165</sub> mixtures enhance VEGFR-2 (Tyr-951) phosphorylation.** Human bone marrow endothelial cells without cell surface HS were incubated with PlnDI/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) PlnDI/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; ( $\gamma$ ) different from VEGF<sub>165</sub>; ( $\phi$ ) different from PlnDI+VEGF<sub>165</sub>.  $p < 0.05$ .





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 PInDI has the capacity to bind and modulate the activity of VEGFR-2 directly, we performed PInDI binding studies against immobilized VEGFR-2, and NRP-1. Outcomes from these studies suggest PInDI-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 PInDI-HS chain binding to NRP-1 occurs via its heparin binding domain [15]. In contrast, PInDI 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 presence of VEGF<sub>165</sub> enhances PInDI binding to VEGFR-2, suggesting the formation of a complex between PInDI/VEGF<sub>165</sub>/VEGFR-2 is possible. Our observations also suggest that modulation of VEGFR-2 signaling by PInDI may involve complex interactions with more than one ligand.

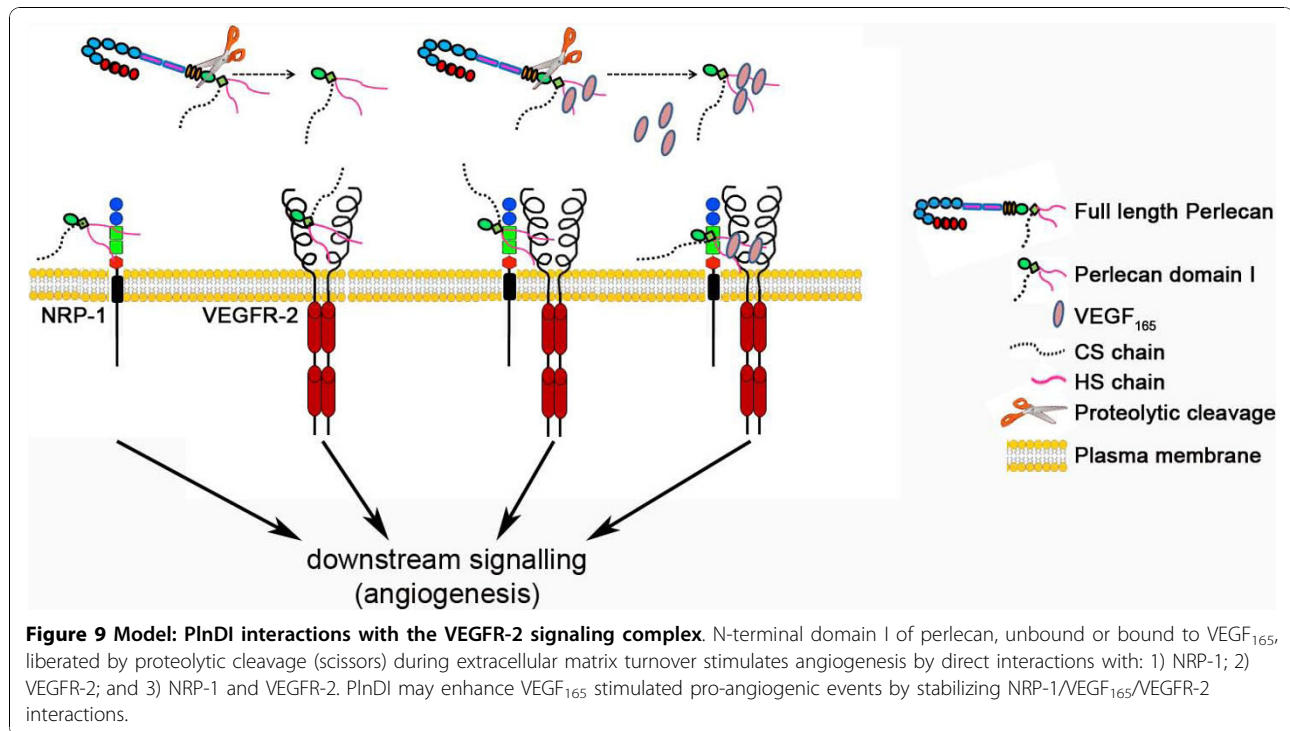
### Conclusion

The findings presented herein demonstrate exogenous, soluble, recombinant PInDI is sufficient to bind and modulate the activity of the VEGFR-2 signaling complex via HS interactions, *in vitro*. Moreover, PInDI 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 PInDI may impact angiogenic events in the absence or presence of VEGF<sub>165</sub>. PInDI unbound or bound to VEGF<sub>165</sub> 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 VEGF<sub>165</sub>, PInDI-HS may bind to NRP-1, VEGFR-2, or support complex formation with both to signal downstream angiogenic events. When VEGF<sub>165</sub> is present PInDI interactions with NRP-1 and VEGFR-2 are optimized, leading to enhanced downstream signaling and angiogenesis.

### Methods

#### Materials

Recombinant human VEGF<sub>165</sub>, VEGFR-2, NRP-1, and anti-VEGF<sub>165</sub> monoclonal antibodies were procured from R&D systems, Inc. (Minneapolis, MN). Growth factor reduced Matrigel was purchased from BD Biosciences (San Jose, CA). Goat polyclonal antibodies to GAPDH were purchased from Genscript (Piscataway,



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 cultures 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 electrophoresed 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  $\mu$ l of PBS containing 4 mM  $\text{CaCl}_2$  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  $\mu$ g) untreated, or pre-digested with a heparinase cocktail and/or chondroitinase ABC was pre-incubated with 20 ng of VEGF<sub>165</sub> 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 VEGF<sub>165</sub> was detected with anti-VEGF<sub>165</sub> antibodies (1  $\mu$ g/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 VEGF<sub>165</sub> 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  $\mu$ l/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  $\mu$ g/ml), untreated or pre-digested with a heparinase cocktail and/or chondroitinase ABC, heparin (4.0  $\mu$ g/ml), VEGF<sub>165</sub> (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  $\mu$ m 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  $\text{Na}_3\text{PO}_4$ , 25 mM NaF, and 1 mM  $\text{Na}_3\text{VO}_4$ , protease inhibitors (Roche Diagnostics, Lewes, UK)], and total protein concentrations determined (micro BCA assay). For Western blotting, 30  $\mu$ g 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  $\mu$ g/well) with or without VEGF<sub>121</sub>, VEGF<sub>165</sub>, or heparin [0.01-1000  $\mu$ g/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 tetramethylbenzidine (KPL; 100  $\mu$ l/well) for 10 min under gentle agitation. Color development was stopped with 50  $\mu$ L of 0.5 N  $\text{H}_2\text{SO}_4$ . 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 disaccharides released from PlnDI following digestion with heparinase cocktail were analyzed as above. Commercially prepared bovine tracheal CS disaccharide standards (ΔDi-0 S, ΔDi-4 S and ΔDi-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 presented as means ± 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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### Author details

<sup>1</sup>Department of Orthopaedics and Rehabilitation, Penn State College of Medicine, Hershey, Pennsylvania 17033, USA. <sup>2</sup>Department of Biological Sciences, Center for Translational Cancer Research, University of Delaware, Newark, Delaware 19716, USA.

### 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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