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Perlecan regulates developmental angiogenesis by modulating the VEGF-VEGFR2 axis

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Abstract

Using the zebrafish, we previously identified a central function for perlecan during angiogenic blood vessel development. Here, we explored the nature of perlecan function during developmental angiogenesis. A close examination of individual endothelial cell behavior revealed that perlecan is required for proper endothelial cell migration and proliferation. Because these events are largely mediated by VEGF-VEGFR2 signaling, we investigated the relationship between perlecan and the VEGF pathway. We discovered that perlecan knockdown caused an abnormal increase and redistribution of total VEGF-A protein suggesting perlecan is required for the appropriate localization of VEGF-A. Importantly, we linked perlecan function to the VEGF pathway by efficiently rescuing the perlecan morphant phenotype by microinjecting VEGF-A₁₆₅ protein or mRNA. Combining the strategic localization of perlecan throughout the vascular basement membrane along with its growth factor-binding ability, we hypothesized a major role for perlecan during the establishment of the VEGF gradient which provides the instructive cues to endothelial cells during angiogenesis. In support of this hypothesis we demonstrated that human perlecan bound in a heparan sulfate-dependent fashion to VEGF-A₁₆₅. Moreover, perlecan enhanced VEGF mediated VEGFR2 activation of human endothelial cells. Collectively, our results indicate that perlecan coordinates developmental angiogenesis through modulation of VEGF-VEGFR2 signaling events. The identification of angiogenic factors, such as perlecan, and their role in vertebrate development will not only enhance overall understanding of the molecular basis of angiogenesis, but may also provide new insight into angiogenesis-based therapeutic approaches.

Keywords

Perlecan; Heparan sulfate proteoglycan; VEGF; VEGF receptor; Angiogenesis; Endothelial cell; Zebrafish

1. Introduction

The vascular basement membrane encompassing various collagens, laminins and perlecan serves not only a structural (Yurchenco et al., 2004) but also a signaling (Ramirez and Rifkin,

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2003) function. A perlecan-mediated growth factor response spans the contexts of development and disease (Wight et al., 1992; Iozzo, 1994; Iozzo et al., 1994; Hassell et al., 2003; Iozzo, 2005; Whitelock et al., 2008). Mutations in the *C. elegans unc-52* perlecan ortholog disrupt muscle organization (Rogalski et al., 1993; Mullen et al., 1999) and disturb distal tip cell migration via influence on FGF¹, TGF β and Wnt-like signaling (Merz et al., 2003). *Drosophila* perlecan, encoded by *trol*, regulates neuroblast proliferation through the FGF and Hh pathways (Voigt et al., 2002; Park et al., 2003). Recently *trol* has been linked to other growth factor signaling pathways functioning either during brain development by TGF and Wnt, or perhaps regulating plasmotocyte proliferation by VEGF/PDGF (Lindner et al., 2007). The perlecan-null mice exhibit a complex phenotype characterized at one level by reduced chondrocyte proliferation, most likely the result of altered growth factor signaling (Costell et al., 1999; Arikawa-Hirasawa et al., 1999). Recent evidence also links murine perlecan to regulating floor plate Shh and forebrain development (Girós et al., 2007). Beyond embryonic development, perlecan-mediated growth factor modulation has been associated with human prostate cancer (Datta et al., 2006b). Essentially perlecan binding to Shh promotes Shh downstream signaling and supports prostate cancer cell growth (Datta et al., 2006a).

The widespread developmental expression (Carson et al., 1993; Handler et al., 1997) and its complex modular structure (Iozzo and Murdoch, 1996; Iozzo, 1998; Farach-Carson and Carson, 2007) suggest that perlecan is involved in a number of physiological and pathological events. Indeed, perlecan is implicated in lipid catabolism (Fuki et al., 2000), epidermal formation (Sher et al., 2006), chondrogenesis (SundarRaj et al., 1995; French et al., 2002), vascular injury and thrombosis (Nugent et al., 2000; Kinsella et al., 2003), atherosclerosis (Tran-Lundmark et al., 2008), and cancer growth and invasion (Cohen et al., 1994; Iozzo et al., 1997; Mathiak et al., 1997). Perlecan-growth factor interactions are mediated through the perlecan protein core or the attached heparan sulfate chains (Iozzo and San Antonio, 2001; Smith et al., 2007; Whitelock et al., 2008). The heparan sulfate chains are required for HS-binding growth factor signaling and influence the distribution or movement of growth factors (Nakato and Kimata, 2002). The pivotal role of the perlecan HS chains to growth factor response has been evidenced by the HS-deficient perlecan mice. These transgenic animals (*Hspg2*^{A3/A3}) harbor a partial deletion of perlecan domain I (exon 3), thereby losing the HS attachment sites (Rossi et al., 2003). HS deficiency was linked to decreased matrix binding of FGF-2, which correlated with increased smooth muscle cell proliferation *in vitro* and *in vivo* (Tran et al., 2004). The perlecan HS-deficient mice also exhibited delayed wound healing as well as impaired tumor growth and angiogenesis induced by FGF-2 (Zhou et al., 2004). Antisense strategies targeting the expression of perlecan protein core cause a marked suppression of tumor growth, angiogenesis, and an attenuated response to FGF-2 in several cell systems (Sharma et al., 1998; Aviezer et al., 1997). Perlecan knockdown in prostate cancer cells has also been shown to disrupt *in vitro* responses to VEGF-A and FGF-2, both HS-binding growth factors (Savoré et al., 2005).

Analysis of the HS-modifying enzymes in zebrafish also supports the relationship between HS and growth factor modulation (Cadwallader and Yost, 2006b; Cadwallader and Yost, 2006a; Cadwallader and Yost, 2007). Investigation of zebrafish heparan sulfate 6-O sulfotransferase revealed that HS6ST was required for muscle and angiogenic vascular development (Bink et al., 2003; Chen et al., 2005). Interestingly HS6ST function, through an interaction with VEGF, is essential for branching morphogenesis of the developing caudal vein (Chen et al., 2005).

¹**Abbreviations:** FGF, fibroblast growth factor; TGF, transforming growth factor; Hh, hedgehog; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; Shh, sonic hedgehog; GAG, glycosaminoglycan; HS, heparan sulfate; HS6ST, heparan sulfate 6-O sulfotransferase; HSPG, heparan sulfate proteoglycan; VEGFR2, vascular endothelial growth factor receptor 2; ISV, intersegmental vessel; DLAV, dorsal longitudinal anastomotic vessel; SIV, sub-intestinal vessel; DA, dorsal aorta; PCV, posterior caudal vein; DIC, differential interference contrast; PLC γ 1, phospholipase C gamma-1; HCAEC, human coronary artery endothelial cell; HUVEC, human umbilical vein endothelial cell.

The heparin-binding VEGF-A actually serves as a ligand for 6-O sulfated heparan sulfate (Ono et al., 1999). Accordingly, the binding is essential for vessel branch establishment and for influencing the spatial restriction of VEGF which regulates branch pattern (Ruhrberg et al., 2002). Perlecan binding to growth factors may establish a morphogen gradient supporting key events such as those alluded to during vessel guidance (Siekman and Lawson, 2007; Hellstrom et al., 2007; Leslie et al., 2007). Perlecan can also serve as a sink for various growth factors which, in a context-dependent manner, could favor or disfavor receptor activation and downstream events (Aviezer et al., 1994). Along these same lines heparanase or proteolytic cleavage of the core could generate functional growth factor complexes (Whitelock et al., 1996). A similar concept was presented in hepatoblastoma xenografts which exhibited initial tumor regression and angiogenesis as a result of VEGF therapy, but eventual recurrence associated with vessel recovery and an increase in both perlecan and heparanase expression (Kadenhe-Chiweshe et al., 2008). Essentially perlecan was sequestering and heparanase was releasing VEGF in the tumor vessel microenvironment which ultimately favored VEGFR2 activation and vessel survival (Kadenhe-Chiweshe et al., 2008).

The purpose of the current study was to further explore perlecan function within the context of angiogenesis and growth factor biology. Given the nature of perlecan function, we hypothesized perlecan may modulate VEGF-VEGFR2 activation as a means to coordinate developmental angiogenesis.

2. Results

2.1. A closer examination: perlecan is required for angiogenic blood vessel development

Perlecan knockdown significantly inhibits angiogenic blood vessel development throughout the trunk and tail (Fig. S1). Angiogenic sprouts, the intersegmental vessels (ISVs), emerge from the dorsal aorta but fail to extend past the region of the notochord to form a complete dorsal longitudinal anastomotic vessel (DLAV) along the dorsal side of the embryo (Zoeller et al., 2008). Live DIC video microscopy revealed these vessel sprouts are largely non-lumenized, non-functional vessels unable to carry flow.

Normal angiogenic blood vessel development of the ISVs in the trunk and tail involves a number of coordinated cell behaviors, among four to six cells, that include sprouting, migration, proliferation, establishment of cell-cell junctions and lumen formation in order to form a complete and functional circulatory network (Isogai et al., 2001; Childs et al., 2002; Isogai et al., 2003; Blum et al., 2008). Our observations of perlecan morphant vasculature indicate that angiogenic sprouting is initiated and occurring at proper intervals, but the fact that the sprouts fail to continue suggests perlecan knockdown may interfere with the subsequent angiogenic cellular events described above.

To determine the role of perlecan in endothelial cell behavior, we examined the consequences of perlecan knockdown on vascular development in *Tg(fli1a:nuclearEGFP)^{y7}* zebrafish embryos (Siekman and Lawson, 2007). Previous experiments utilized the *Tg(fli1:EGFP)^{y1}* zebrafish line which express cytoplasmic GFP under the control of *fli1* a vascular specific marker- fluorescently labeling the entire zebrafish vascular network and permitting *in vivo* analysis of vascular development during real time (Lawson and Weinstein, 2002). The *Tg(fli1a:nEGFP)^{y7}* were engineered in nearly the same manner but harbor nuclear localized GFP expression, permitting analysis of individual endothelial cell behavior *in vivo* and over real time (Siekman and Lawson, 2007). Analysis of perlecan knockdown in the *Tg(fli1a:nEGFP)^{y7}* was capable of further defining the nature of abnormal ISV development at the individual cell level, which includes assessing sprout cell number, migratory behavior and cell division in the absence of perlecan.

We found perlecan morphant ISVs contained a significantly less number of endothelial cells throughout the ISV region when compared to matched control embryos (Fig. 1). On average, the 2 dpf perlecan morphants displayed four endothelial cell nuclei per ISV region (Fig. 1B) versus eight endothelial cell nuclei per ISV region in controls (Fig. 1A). Given the abnormal nature of ISV cell behavior in the morphant embryos, we hypothesized that perlecan supports the migratory and or proliferative events necessary for proper angiogenic blood vessel development. Since such proangiogenic events are controlled by the vascular endothelial cell growth factor and its receptor, we investigated a link between perlecan and VEGF-VEGFR2.

2.2. Perlecan is required for the proper localization of VEGF-A

We hypothesized that perlecan would sequester VEGF, thereby regulating VEGF positional distribution or availability and functional activity through VEGFR during angiogenesis. Whole-mount immunohistochemistry with anti-VEGF-A, showed that VEGF was primarily expressed in the fin and neck region in 2–3 dpf control embryos (Fig. 2A–C). To our surprise, the amount of VEGF was markedly increased and abnormally distributed in the perlecan morphants (Fig. 2E,F). Specifically, in the perlecan morphants VEGF accumulated diffusely in the dorsal and ventral regions of the future caudal fin (Fig. 2E–F, arrows) and in the hindbrain (Fig. 2E–F, dotted arrows). Given the nature of the perlecan morphant angiogenic phenotype we suggest that the VEGF deposits are largely nonfunctional in the absence of perlecan. We further explored this finding by performing additional immunostaining using cross-sections through the trunk (Fig. 3). We observed VEGF as significant punctuate deposits around regions of the developing notochord and spinal cord in the perlecan morphants (Fig. 3B,C) compared to matched control embryos (Fig. 3A). These data support the original observations at the whole organism level and provide new insight regarding abnormal VEGF localization to key regions.

2.3. Perlecan influences VEGF-A protein levels

To biochemically assess our VEGF-A immunostaining data, we performed immunoblotting on protein samples derived from pooled 2 dpf control or morphant embryos ($n = 26$ each, Fig. S2). Immunoblotting with anti-VEGF revealed that perlecan morphants exhibited elevated levels of total VEGF-A when compared to control embryos (Fig. 3D, bottom). The multiple band patterns represent multiple VEGF-A isoforms and or dimer formation. Immunoblotting with anti-acetylated tubulin served as a load control (Fig. 3D, top). Our biochemical analysis of the perlecan morphants indicates that the abnormal topographical distribution of VEGF-A (Figs. 2 and 3A–C) was associated with an abnormal increase in VEGF-A protein levels (Fig. 3E).

2.4. VEGF-A can partially rescue the perlecan morphant phenotype

Next, we tested whether microinjection of either VEGF-A₁₆₅ protein or mRNA could rescue the phenotype evoked by knockdown of endogenous perlecan. To this end, embryos were injected with MO-DI alone or in combination with VEGF-A₁₆₅ (1.25 ng/embryo). In three independent experiments, VEGF was capable of rescuing the curved-body phenotype as well as the structure and organization of the ISV/DLAV (Fig. 4). We obtained a similar rescue of the vascular phenotype using zebrafish VEGF-A₁₆₅ mRNA, although at a 40% rescue response (Fig. S3). Microinjection of VEGF-A₁₆₅ alone did not induce any observable phenotype (data not shown). Thus, perlecan morphants can be partially rescued by VEGF-A, indicating that perlecan acts upstream of the VEGF-VEGFR2 signaling axis.

2.5. Perlecan binds VEGF-A via the HS side chains

Our VEGF rescue experiments indicated that perlecan acts upstream, at the level of VEGF-VEGFR, within the VEGF signaling pathway. We hypothesized that perlecan directly regulates VEGF during angiogenic blood vessel development. To gain further insight and to support a

direct mechanism of action, we examined perlecan-VEGF protein-protein interaction and the relationship between perlecan and VEGF function. Using overlay assay we tested human VEGF-A and FGF-2, two established HS-binding proteins, and as positive control for the protein core we used recombinant progranulin, a secreted growth factor which specifically binds to domain V/endorepellin of human perlecan (Gonzalez et al., 2003). The different proteins were absorbed onto nitrocellulose membranes using scalar dilutions, followed by overlay with perlecan derived from human coronary artery endothelial cells. Immunoblotting with anti-perlecan/domain V revealed that perlecan bound VEGF-A, FGF-2 and progranulin (Fig. 5A). We found that pre-incubation with excess heparin, prior to overlay with perlecan, inhibited perlecan binding to VEGF-A and FGF-2 but not to progranulin (Fig. 5B). Our results support a perlecan protein core mediated interaction with progranulin and indicate that perlecan binds both FGF-2 and VEGF-A via its HS side chains. We propose that the perlecan-VEGF interaction is required for VEGF function.

2.6. Perlecan enhances VEGFR2 activation by VEGF-A

Next, we investigated the influence of a perlecan-VEGF interaction on endothelial cell function *vis-à-vis* activation of VEGFR2, the major signaling receptor tyrosine kinase which mediates multiple events during the angiogenic cascade in response to VEGF-A ligands (Olsson et al., 2006; Dai and Rabie, 2007). By immunoblotting, we analyzed human endothelial cell VEGFR2 phosphorylation status in response to a 2 min or 5 min stimulation with human perlecan or VEGF-A alone, versus a combination of the two. Interestingly, we found that a pre-incubation of perlecan plus VEGF-A followed by a short application to the endothelial cells evoked stronger VEGFR2 phosphorylation compared to either used alone (Fig. 6). As shown in Fig. 6, a 5-min stimulation using perlecan plus VEGF induced a seven fold increase in VEGFR2 phosphorylation in relation to VEGF stimulation alone. Our results suggest that the interaction between perlecan and VEGF-A promotes VEGFR2 signaling during angiogenesis.

3. Discussion

Our previous work applied the zebrafish animal model to dissect perlecan function. These studies identified a central role for perlecan during muscle and vascular development (Zoeller et al., 2008). Perlecan knockdown largely inhibited angiogenic blood vessel development of the intersegmental vessels, dorsal longitudinal anastomotic vessel and sub-intestinal vessels, while unaffected vasculogenesis of the axial vessels (dorsal aorta and posterior cardinal vein). The vascular phenotype characterized by perlecan knockdown strikingly resembles knockdown of key components of the VEGF signaling pathway. Morpholino-mediated knockdown of zebrafish VEGF-A (Nasevicius et al., 2000), the major VEGF-A receptor VEGFR2 (Habeck et al., 2002; Covassin et al., 2006), and PLC γ 1, the major downstream target of VEGF-VEGFR angiogenic signaling (Lawson et al., 2003), all phenocopy the perlecan morphant vascular phenotype. These observations also suggested perlecan knockdown may interfere with the VEGF-VEGFR signaling cascade during angiogenic blood vessel development. A clear link between perlecan and the VEGF signaling pathway during zebrafish vascular development was established via rescue of the perlecan morphant vascular phenotype with VEGF-A₁₆₅. Utilizing protein or mRNA, we found VEGF-A₁₆₅ could partially rescue the abnormal angiogenic sprouting in the perlecan morphant trunk and tail—supporting a link to perlecan function through VEGF.

Our rescue with VEGF-A suggested perlecan functions upstream of VEGFR2. Accordingly, we predicted the heparan sulfate proteoglycan perlecan was capable of interacting with VEGF-A, a known heparin-binding growth factor. We hypothesized that perlecan binding sequesters VEGF and thereby regulates VEGF positional distribution, availability and functional activity through VEGFR during angiogenesis. Using whole-mount immunohistochemistry we found

that perlecan and VEGF exhibited largely overlapping expression profiles. Interestingly, we have found that lack of endogenous perlecan resulted in the abnormal accumulation and redistribution of VEGF, supporting a perlecan-VEGF positional role. We predict that these VEGF deposits are largely non-functional, non-utilized growth factors. Interestingly, the abnormal increase in VEGF may also represent a compensatory effect whereby the embryo would try to overcome inhibition of angiogenesis by upregulating a key component of this biological process. Accordingly, hypoxic conditions induce VEGF to promote angiogenesis (Nomura et al., 1995). Along these same lines, we could predict that the embryo alone cannot compensate but an excess of VEGF-A₁₆₅ mRNA or protein, supplied via rescue, could be possibly sufficient to evoke VEGFR2 activation and ultimately rescue of the phenotype. Intriguingly, the upregulation of VEGF might actually represent a means to induce perlecan synthesis in the perlecan morphant embryos. VEGF-A₁₆₅ has been identified to increase perlecan expression in human brain microvascular endothelial cells (Kaji et al., 2006).

We confirmed the binding between perlecan and VEGF-A, and defined the interaction as largely mediated via the heparan sulfate side chains. The functional consequences of perlecan-VEGF complexes were characterized as enhancing VEGFR2 phosphorylation status. The role of perlecan HS is supported by heparin-mediated enhanced VEGFR2 phosphorylation as well (Ashikari-Handa et al., 2005). VEGFR2 activation in response to perlecan-VEGF would favor downstream signaling events. Accordingly, endothelial cell migration and proliferation would proceed in support of the angiogenic cascade. Combined these results suggest HSPG perlecan serves as a crucial growth factor-interacting partner which can influence growth factor receptor signaling.

Our results establish perlecan function through the coordinated modulation of VEGFA-VEGFR2 signaling. Combined our *in vivo* and *in vitro* data indicate that perlecan binds and localizes VEGF-A in a tissue-specific manner. Furthermore, perlecan/VEGF-A interaction enhances VEGFR2 activity thereby promoting endothelial cell migration and proliferation during vascular development. Perlecan function within this context represents one aspect, out of the multiple and complex events, contributing to developmental angiogenesis.

4. Experimental procedures

4.1. Generation and analysis of the perlecan morphants

Perlecan knockdown was achieved by utilizing a translation blocking morpholino (MO-DI; Gene Tools, LLC) as previously described (Zoeller et al., 2008). All wild-type and vascular transgenic *Tg(fli1:egfp)^{y1}* (Lawson and Weinstein, 2002), *Tg(fli1a:negfp)^{y7}* (Siekman and Lawson, 2007), *Tg(vegfr2:g-rcfp)* (Cross et al., 2003) embryos, were housed in the zebrafish facility of Thomas Jefferson University, cared for according to standard practice and imaged on the platforms previously described (Zoeller et al., 2008).

4.2. Analysis of zebrafish VEGF-A protein levels

For immunoblotting: Total protein was extracted in RIPA buffer from pooled 2 dpf perlecan morphant and matched control embryos. A portion of the lysate was subjected to standard SDS-PAGE and transfer to nitrocellulose membranes. Immunoblotting was performed using anti-VEGF (A-20:sc-152, Santa Cruz) or anti-acetylated tubulin (T7451, Sigma), followed by donkey anti-rabbit HRP (GE Healthcare) or goat anti-mouse HRP (Pierce) and detection by ECL (Pierce). For immunohistochemistry: Whole-mount immunostaining was performed on groups of morphant and matched control 2–3 dpf embryos using anti-VEGF (A-20:sc-152, Santa Cruz) as described previously (Zoeller et al., 2008). Cross sections were prepared by standard cryosection, immunostaining was performed by blocking in 5% FBS, followed by

incubation with anti-VEGF (A-20:sc-152, Santa Cruz) and detection by anti-rabbit Rhodamine, both in 1% FBS.

4.3. Rescue with VEGF-A₁₆₅ protein or RNA

Vascular transgenic 1-cell stage embryos were microinjected with either MO-DI or VEGF-A₁₆₅ alone, or a combination of the two in a dose equal to the injection of either component alone. Recombinant human VEGF-A₁₆₅ (293-VE/CF, R&D Systems) was used similar to previous applications (Ma et al., 2007; Serbedzija et al., 2000). Zebrafish VEGF-A₁₆₅ mRNA (pCS2:vegfl65 kindly provided by N. Lawson, (Lawson et al., 2002)) was prepared by *in vitro* transcription using SP6 mMACHINE (Ambion). Rescue was assessed at 2 dpf by analyzing the embryos' gross and vascular phenotype.

4.4. Protein-protein interaction by perlecan overlay assay

Human perlecan was immunoaffinity purified from the secretions of human coronary arterial endothelial cells using an affinity column containing a monoclonal antibody against domain III of perlecan protein core (Murdoch et al., 1994) using protocols described before (Whitelock et al., 1999; Whitelock and Iozzo, 2002). Recombinant human VEGF-A₁₆₅ (293-VE/CF, R&D Systems), recombinant human FGF-2 or progranulin (Gonzalez et al., 2003) were spotted by slot blot onto nitrocellulose membranes. Membranes were briefly washed in PBS followed by blocking for 30 min. in 5% milk. Membranes were overlaid with human coronary artery endothelial cell (HCAEC) derived perlecan (Whitelock and Iozzo, 2002; Whitelock et al., 1999) for ~4 h in PBS at room temperature. Membranes were briefly washed in PBS prior to detection of a positive binding interaction by standard immunoblotting using anti-perlecan/ domain V (Bix et al., 2004), donkey anti-rabbit HRP (GE Healthcare) and ECL (Pierce). For heparin competition experiments, blots were pre-incubated with excess heparin (10 µg/ml) for 1 h prior to similar overlay (~2 h) and detection as described above.

4.5. Analysis of endothelial cell VEGFR2 activation

Early passage HUVEC were grown to confluency in complete media according to standard protocol. Monolayers were stimulated for 2 min. or 5 min. with either HCAEC perlecan (~1 µg/ml) or human recombinant VEGF-A₁₆₅ (10 ng/ml; 293-VE/CF, R&D Systems) alone or a combination of the two (~1 µg/ml perlecan plus 10 ng/ml VEGF-A₁₆₅, pre-incubated for 1 h at room temperature). Total cell lysates were extracted in RIPA buffer and subjected to standard SDS-PAGE and transfer to nitrocellulose membranes. Immunoblotting was performed using phospho-VEGFR2 (Y951) antibody (2471, Cell Signaling), anti-FLK-1/VEGFR2 (C-1158:sc-504, Santa Cruz) or anti-acetylated tubulin (T7451, Sigma), followed by donkey anti-rabbit HRP (GE Healthcare), anti-rabbit IRDye 800CW (Li-COR) or goat anti-mouse HRP (Pierce) respectively.

4.6 Statistical analysis

Experiments were run in triplicates and were statistically analyzed by Student's *t* test using Sigma Stat 10.0 (SPSS). Differences were considered significant at *P* < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Perlecan knockdown influences endothelial cell number. Vascular analysis of control (A) or perlecan morphant (B) *Tg(fli1a:nEGFP)^{y7}* zebrafish embryos. Notice the abnormal formation of ISVs (arrows) and the lack of a DLA in the perlecan morphants. (C) Endothelial cell number was assessed by counting the endothelial cell nuclei present per intersegmental vessel region in 2 dpf control and morphant embryos. Control embryos typically displayed eight endothelial cells/ISV region compared to perlecan morphants which typically displayed four cells in the comparable area. Data summarize the endothelial cell counts from a total of 40 ISV regions for controls and a comparable 40 ISV regions for morphants. The forty counts were derived from four separate embryos, with 10 ISV regions assessed in each (***) $P < 0.001$. Scale bar, 250 μ m.

Fig. 2.

Perlecan knockdown results in the abnormal redistribution and accumulation of VEGF protein in morphant embryos. Compare the VEGF whole-mount immunostaining between 2–3dpf control (A–D) and MO-DI (E–G) embryos. Notice the accumulation and abnormal localization of VEGF epitopes in the dorsal and ventral soft tissues of the future caudal fin (red arrows) and an increase in the hindbrain region (dotted red arrows). Panels (D) and (G) are staining with secondary antibody alone. All images are left-side views with dorsal up and anterior to the left. Scale bars, 600 μ m (A, D, G) and 305 μ m (B, C, E, F).

Fig. 3. Perlecan knockdown in zebrafish results in an increase in VEGF protein levels. Cross-section analysis revealed regions of abnormal VEGF deposits in perlecan morphant embryos. Immunostaining identified abnormal VEGF aggregates surrounding the notochord (NC, arrows in B and C) and within the region of the spinal cord (SC) in 2 dpf perlecan morphant embryos (B and C). Panel A represents a frozen cross-section from a matched 2 dpf control embryo. Immunostaining was performed with anti-human VEGF-A and visualized by anti-rabbit-Rhodamine (red) and counterstained with DAPI (blue). Scale bar, 90 μ m. (D) Protein extracted from 2 dpf control ($n = 26$) and MO-DI ($n = 26$) embryos was assayed for VEGF expression levels by immunoblotting with anti-VEGF antibody and anti-acetylated tubulin as a load control. Notice the significant increase in total VEGF in the perlecan morphants. The various immunoreactive bands correspond to monomers, dimers and higher-order complexes of VEGF. (E) Numerical estimation of the data presented in D, $n = 3$ sample runs (** $P < 0.01$). Blots were quantified using the ImageJ 1.41o software package (NIH).

Fig. 4. VEGF-A₁₆₅ protein can rescue the morphant phenotype induced by MO-DI perlecan knockdown. Panels (A,B) are live bright field and corresponding GFP composite images of a representative control (top), MO-DI (middle) and rescue (MO-DI + VEGF-A₁₆₅, bottom) sample. Note the clear rescue of both the muscle and vascular phenotype by VEGF-A₁₆₅, as compared to MO-DI alone. Microinjection of VEGF-A₁₆₅ alone did not result in any observable phenotype (data not shown). All images are left-side views of 3dpf *Tg(vegfr2:gfp)* embryos with dorsal up and anterior to the left. Scale bar, 500 μ m. The graph displayed in (C) represents the mean $\pm$ S.E.M. of three individual experiments, with rescue screening based upon embryos exhibiting normal vascular development (** P <0.01; *** P <0.001).

Fig. 5.

Perlecan binds VEGF-A in overlay assays. (A) Human coronary artery endothelial cell derived perlecan binds VEGF-A, FGF-2 and progranulin. Proteins were slot-blotted onto nitrocellulose membranes at the indicated amounts, blocked for 30 min in 5% milk, followed by ~4 h overlay with perlecan (125 ng/ml). Perlecan binding was revealed by immunodetection with anti-human perlecan domain V. (B) Heparin competition inhibits perlecan binding to VEGF-A and FGF-2 but not progranulin. The blots presented in (A) were stripped, examined for residual signal by ECL, blocked in 5% milk for 30 min, followed by pre-incubation with heparin (10 μ g/ml) for ~1 h and subsequent overlay with perlecan (125 ng/ml) for 2 h. Perlecan binding was examined as described in A.

Fig. 6.

Perlecan enhances VEGF-A induced phosphorylation of VEGFR2. Human endothelial cells were exposed to human perlecan, human VEGF-A₁₆₅ or a combination of both for a 2 min or 5 min stimulation. Total protein lysates were immunoblotted for phospho-VEGFR2 (Y951), total VEGFR2 and acetylated tubulin as a load control. The combination of perlecan plus VEGF-A was capable of enhancing VEGFR2 activation status (lanes 4 and 8) compared to VEGF-A (lanes 3 and 7) or perlecan (lanes 2 and 6) alone. Lanes 1 and 5 represent endothelial cell cultures incubated with only media.