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Review

Perlecan: how does one molecule do so many things?

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Abstract. Perlecan is a large multi-domain extracellular matrix proteoglycan that plays a crucial role in tissue development and organogenesis. In vertebrates, perlecan functions in a diverse range of developmental and biological processes, from the establishment of cartilage to the regulation of wound healing. How can a single molecule modulate such a wide variety of processes? We suggest that perlecan employs the same basic mechanism, based on interactions with growth factors, morphogens and matrix proteins, to regulate each of these processes and that the local extracellular environment determines the function of perlecan and consequently its downstream effects on the structure and function of the organ. We discuss this hypothesis in relation to its role in three major vertebrate developmental processes: angiogenesis, chondrogenesis and endochondral ossification.

Keywords. Perlecan, angiogenesis, chondrogenesis, cartilage, heparan sulfate, FGF, VEGF.

Introduction

Perlecan is a large (>400 kDa) multi-domain extracellular matrix (ECM) proteoglycan that has been evolutionarily conserved in organisms as distinct as nematodes, fruit flies and mammals [1, 2]. Perlecan was initially isolated from a mouse Engelbreth-Holm-Swarm (EHS) tumor [3] and was subsequently found to be expressed in nearly all basement membranes as well as mesenchymal organs and connective tissues [4]. The protein core consists of five domains which share homology with other molecules involved in nutrient metabolism, cell proliferation and adhesion, including laminin, low-density lipoprotein (LDL) receptor, epithelial growth factor (EGF) and neural-cell adhesion molecule (N-CAM). Important to note is that mouse perlecan differs from human perlecan in that it has a significantly smaller domain IV (N-CAM repeats) as well as the presence of a cell-binding RGD sequence in domain III [1]. A detailed discussion of the structural characteristics and expression patterns of the perlecan molecule are available elsewhere [1, 4] and the reader is directed to these reviews and those by Jiang and Couchman [5] and Iozzo [6, 7]. Within the protein core there are numerous sites for O-linked glycosylation as well as four potential sites for heparan sulfate (HS)/chondroitin sulfate (CS) chain attachment: three on domain I and one on domain V. These carbohydrate chains, as well as the domains of the protein core, are known to interact with a wide range of biological molecules as shown in Figure 1. It should be noted that most research to date has focused on the role of the HS chains attached to domain I of perlecan, since these structures are known to regulate growth factor activity by serving as reservoirs (sequestering and storing growth factors in the ECM), and as low-affinity co-receptors [reviewed in ref. 8].

In vertebrates, perlecan functions in a diverse range of developmental and biological processes from the establishment of cartilage to the regulation of wound healing as well as bone formation. How can a single molecule modulate such a wide variety of effects? In this review, we hypothesize that perlecan employs the same basic mechanism, based on interactions with growth factors, morphogens and matrix proteins (Fig. 1), to regulate each of these processes, and that it is the local environ-

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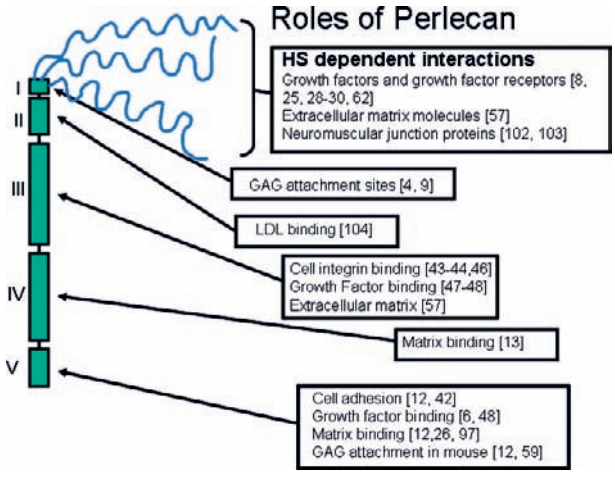

Figure 1. Roles of perlecan. The diverse functions of perlecan are mediated by interactions of perlecan protein core and glycosaminoglycan chains with a wide variety of molecules. The most N-terminal region is domain I which contains three GAG chains represented by the lines drawn in freehand. These chains, which are usually heparan sulfate (HS), have been shown to be involved in many interactions, including those with growth factors, ECM molecules and neuromusculular junction proteins. The protein sequence continues through to the most C-terminal domain V, which may also contain a GAG chain in some instances and has been shown to be important for cell and matrix interactions. The numbers referred to relate to references outlining research on the various functions of perlecan and are included in the bibliography.

ment around the cell that determines the role of perlecan and, consequently, its impact on the final structure and function of the tissue that is generated. We support this conjecture by comparing the function of perlecan protein core and glycosaminoglycan chains in three major vertebrate developmental systems: angiogenesis, chondrogenesis and endochondral ossification, and then demonstrate how this one gene product might be able to co-ordinate, modulate and play a crucial role in tissue development and organogenesis.

Perlecan and blood vessel development

In mice perlecan expression was identified in early development [embryonic day (E) 10.5] with deposition in tissues undergoing vasculogenesis, including heart, pericardium and major blood vessels [4, 9]. Perlecan-null embryos display abnormalities in cardiac development, with discontinuities in the myocardial wall resulting in hemorrhage [10], malpositioning of the great arteries, and outflow tract obstruction caused by abnormally abundant mesenchymal cells expressing smooth muscle cell (SMC) alpha-actin [9]. Surprisingly, these animals do not show vascular pathologies, suggesting that perlecan may be involved in development of the heart rather than in the de novo formation of blood vessels. Perlecan may also play a key role in maintaining basement membrane integrity in mechanically stressed cells, because in the knockout animal, the basement membrane matrix becomes unstable and begins to develop discontinuities over time [10]. The mechanism for this is unclear; however, Davis and Senger [11] suggest that this may be due to loss of perlecan, which in turn may render the laminins more susceptible to proteolysis. The interaction of perlecan with the ECM molecules nidogen-1 and -2, type IV collagen and fibronectin [12–15] may also be required for basement membrane stabilization.

Perlecan has been demonstrated to have a role in angiogenesis. Angiogenesis refers to the formation of new blood vessels via extension or remodeling of existing blood vessels as opposed to vasculogenesis, where blood vessels develop from progenitor cells that coalesce and differentiate to form vessels. Using the rabbit ear chamber method, Aviezer et al. [16] showed that perlecan isolated from EHS tumor coated onto alginate beads induced capillary and vessel formation, most likely through binding and activating the angiogenic factor fibroblast growth factor 2 (FGF2).

Further studies using tumor growth as an angiogenic model confirmed the involvement of perlecan in angiogenic processes. In human breast [17], 1iver [18], ovarian [19] colon and prostate carcinomas [reviewed in ref. 6], high levels of perlecan are detected around newly formed blood vessels. Several knockdown studies in tumor cells further demonstrated perlecan produced by these cells to be a mediator of tumor angiogenesis. Savore et al. [20] showed that ribozyme-mediated knockdown of perlecan in metastatic prostate cells resulted in diminished vascularization and tumor growth *in vivo*. Growth and neovascularization of tumor xenografts induced by human colon carcinoma cells and of tumor allografts induced by highly invasive mouse melanoma cells were suppressed when cells were transfected with antisense perlecan [21]. Surprisingly, inhibitory effects have also been observed upon reduction in perlecan expression. Fibrosarcoma and Karposi sarcoma cells transfected with antisense perlecan exhibited enhanced tumor growth in nude mice [22, 23]. One possible explanation for these seemingly contradictory outcomes is that the cell source of perlecan dictates how angiogenesis is regulated, such that perlecan derived from tumor cells of epithelial origin positively regulates angiogenesis, whereas perlecan from mesenchymal tumor cells negatively regulates vessel formation. This anti angiogenic potential of mesenchymal perlecan is consistent with the recent finding that perlecan derived from primary endothelial cells activates the tumor suppressor PTEN in SMCs *in vitro* and *in vivo* [24]. Such differences in the activity of perlecan in modulating tumor angiogenesis are consistent with the study by Knox et al. [25] which demonstrated that perlecans derived from different cell sources vary in their ability to promote FGF2 signaling.

Cellular processing of perlecan may also result in perlecan fragments with activities that differ from the whole molecule. For example, cleavage of perlecan to produce the antiangiogenic domain V fragment named endorepellin may inhibit angiogenesis [26]. This would be similar to what occurs for the proteoglycan collagen XVIII which is cleaved to produce the antiangiogenic factor endostatin [27]. In addition, given that all angiogenic studies to date have involved tumor-derived perlecan and that tumor-derived perlecan behaves differently from perlecan derived from primary cells in growth factor signaling assays [25], it is also essential to establish a role for non-tumor derived perlecan in blood vessel formation.

How are the angiogenic effects of perlecan mediated?

Role of the HS chains

Perlecans can be decorated with HS, CS or keratan sulfate (KS) [28]; however, perlecan isolated from endothelial cells has so far been shown to contain predominantly HS. Endothelial cell-derived perlecan has been shown to bind the angiogenic factors FGF1, 2 and FGF receptor 1 or 3/FGF2 complexes via the HS chains, as well as to promote FGF signaling *in vitro* [8, 25, 29, 30]. We have found that FGF7 and FGF receptor 2b (FGFR2b) either alone or together in solution bind to endothelial-derived perlecan (Fig. 2a) and that this interaction promotes cell signaling by the FGF7/FGFR2b complex *in vitro* (Fig. 2b). Binding to perlecan was found to be HS dependent, because it was competed effectively with heparin in solution (data not shown). The shape of the curves in Figure 2a is indicative of an interaction involving a complex number of binding sites, and is consistent with the heterogeneic structure of HS chains. In addition, regardless of cell type, cells transfected with antisense perlecan display poor responses to angiogenic HS-binding growth factors *in vitro* including vascular endothelial growth factor A (VEGF-A) [20], FGF2 [20, 23] and hepatocyte growth factor (HGF) [23], suggesting that the HS chains of perlecan are required for growth factor signaling.

More recent evidence in support of a role for perlecan HS in angiogenesis comes from the transgenic mice deficient in HS-modified perlecan. Mice were generated by gene targeting of the perlecan (*Hspg2)* gene to remove exon 3 which contains attachment sites for three HS side chains. These mice showed impaired FGF2-induced angiogenesis in tumor and corneal models [31]. Similar to the perlecan-null embryo, the perlecan-HS-deficient animal exhibited relatively normal vasculogenesis. However, perlecan HS was observed to be required for response to vessel injury, as perlecan-HS-deficient mice displayed delayed wound healing and poor vascularization of wound tissue [31]. The authors suggest that this is due to reduced FGF2-mediated angiogenesis, since this factor plays a critical role in wound healing. These same mice also exhibited increased intimal hyperplasia within the lesions [32]. Perlecan has been shown to suppress SMC replication *in vitro*, thus maintaining SMC quiescence [33]. Recently, Garl et al. [24] proposed a mechanism for this activity: perlecan increases activation of PTEN in SMCs resulting in the inhibition of cell proliferation [24]. This is consistent with the observations of reduced PTEN activation associated with high *in vivo* SMC growth rates [34] and areas of hyperplasia in the developing aorta of perlecan-deficient mouse embryos [24]. Perlecan HS chains may be essential to this activity, because SMCs plated on heparinase-treated perlecan rescues cell proliferation and reduces PTEN activity [24], and the perlecan-HS-deficient SMCs exhibited increased proliferation *in vitro* as well as within surgery-induced lesions *in vivo* [32]. Interestingly, these cells were more responsive to stimulation by FGF2, which implies that perlecan HS chains sequester the growth factor preventing receptor binding and thus

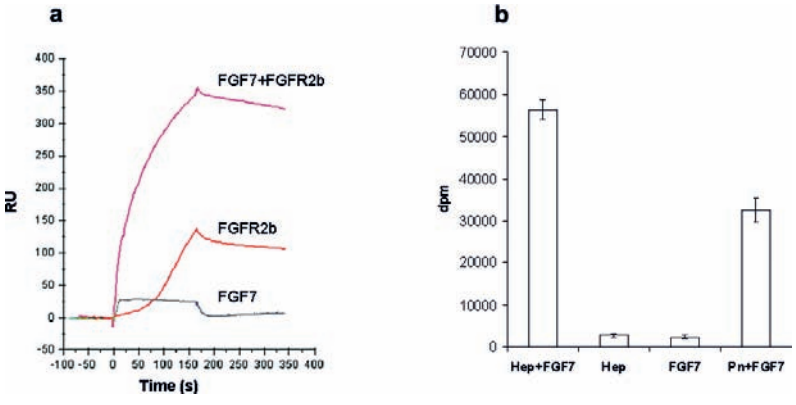

Figure 2. Perlecan binds FGF7 and potentiates FGF7 mitogenic activity *in vitro*. (*a*) Interaction of FGFR2b with endothelial-derived perlecan in either the presence or absence of FGF7. 50 nM FGFR2b was incubated with or without 50nM FGF7 for 5 min at room temperature prior to injection across the surface of a biosensor chip (BIAcore) derivitized with perlecan. RU, response units. (*b*) Proliferation of FGFR2b-expressing Baf32 cells in the presence of perlecan and FGF7. Proliferation was measured via 3H thymine labeling. Assays were performed as described previously [25, 101]. Hep, heparin; Pn, perlecan.

inhibiting mitogenesis. This activity is in contrast to that of the cell surface HSPG syndecan-4, which binds and potentiates FGF2 mitogenic activity in SMCs [35].

ECM degradation is also required for vascular remodeling and various matrix metalloproteinases (MMPs) including membrane bound MMPs (MT-MMPs), MMP-3, MMP-10 and MMP-9 as well as other classes of serine proteinases are involved. Purified perlecan has been shown to be cleaved by stromelysin-1(MMP-3) and this was suggested to lead to the release of FGF2 from the matrix which could then facilitate angiogenesis [36]. MMP cleavage of perlecan has not been investigated in cell-based systems but may provide a mechanism for controlling growth factor diffusion and receptor activation.

Heparan sulfate proteoglycans (HSPGs) are also thought to act as docking stations for some MMPs and their inhibitors, the TIMPs (tissue inhibitors of metalloproteinases). There is limited information on interactions of MMPs with HSPGs or other ECM proteins: however, MMPs have been released from cell culture via heparinases or heparin, and there is evidence for the binding of MMP-2, -7, -9, and -13 to HS in the extracellular space [37]. Of the four TIMPs, TIMP-3 is uniquely localized to the ECM [38] and is sequestered to the pericellular environment by association with HSPGs [39]. Perlecan may be the HSPG involved, as an unpublished study by Saunders et al. demonstrated that TIMP-3 binds perlecan HS [S. Saunders, personal communication] and like perlecan, TIMP-3 is also localized pericellularly. TIMP-3 can inhibit endothelial cell invasion and tube formation in three-dimensional collagen or fibrin matrices [40] as well as inhibit tumor angiogenesis [41]. This activity is most likely via the inhibition of MMPs and ADAMs (a disintegrin and metalloproteinase). Thus perlecan may play a prominent role in both the activation and inhibition of angiogenesis depending on its cellular context, consistent with the expression of perlecan in both vascularized and hypovascularized (i.e. cartilage) tissues.

What is the role of the protein core in angiogenesis?

Given the significant contribution of perlecan to angiogenesis, there is surprisingly very little research on the role of the protein core in blood vessel development other than its role in the modulation of the adhesion of vascular cells [30, 42] and its significant binding of vascular mitogenic growth factors. The protein core of perlecan has been shown to be important in the binding of cells, more than likely via integrin pathways. This has been shown to involve both β1 and β3 integrins [43, 44]; however, the source of perlecan used in these studies was from the EHS tumour, which may have been contaminated with laminin (laminin is well known to interact with cells via β1 integrins). In addition, mouse perlecan contains an RGD consensus sequence within domain III that is not present in the human form. Whether this sequence is involved in cell adhesion is contentious [45, 46].

Although intact perlecan has been shown to bind angiogenic growth factor in an HS-dependent fashion, recombinant domains of perlecan core protein have been shown to interact with a number of angiogenic factors *in vitro* including FGF7 [47] and platelet-derived growth factor (PDGF)-AA and -BB [48]. This suggests that proteolytic processing (or alternative splicing) may produce fragments of the protein core which have their own unique activity. Indeed, an antiangiogenic effect of perlecan has also been observed in the form of endorepellin, an 85-kDa protein derived from the C-terminal domain V of perlecan [49]. Endorepellin has been shown to be antiadhesive for a number of cell types, and to inhibit endothelial cell migration, endothelial tube-like formation within type I collagen matrix, and angiogenesis in chicken chorioallantoic membrane and Matrigel plug assays [49]. Interaction of the terminal laminin-like globular (LG3) domain of endorepellin, the region that possesses most of the biological activity on endothelial cells, with the major cell surface receptor for collagen I, $\alpha 2\beta 1$ integrin, has been shown to result in signaling events leading to disassembly of actin stress fibers and focal adhesions *in vitro* [42]. Although a 26-kDa fragment identical to LG3 has been found in urine of patients with end-stage renal failure, it has not been found in circulating blood or proven to occur in tissues, such that its biological role remains speculative. Although the protease that cleaves endorepellin into the 26-kDa fragment has been identified (BMP-1/Tolloid-like metalloprotease [26]), intact perlecan has not been cleaved to produce this 26-kDa fragment.

Another role for the protein core of perlecan may be to stabilize the newly formed vessel basement membrane. Vascular basement membranes in perlecan knockout mice develop normally; however, they deteriorate in regions with increased mechanical stress such as the contracting myocardium [10]. Perlecan protein core interacts with a number of basement membrane proteins *in vitro* including nidogen-1 and -2, type IV collagen and fibronectin, and these interactions may be required to stabilize and strengthen the network [12–15]. As mentioned earlier, perlecan may also act to stabilize the basement membrane by preventing the proteolysis of laminin. It would be of great interest to investigate further the nature of these interactions both *in vitro* and *in vivo* to assess the extent of the contribution of perlecan protein domains, including domain I and its attached HS, to blood vessel synthesis and maintenance.

Chondrogenesis and bone formation

Perlecan is essential for normal cartilage and bone development in mice and humans, as evidenced by knock-

out studies in mice and the identification and analyses of mutations in the perlecan gene in humans. Perlecan-deficient mouse embryos that survive past embryonic day 10.5 go on to develop severe skeletal dysplasia characterized by shortened/thickened bones and craniofacial abnormalities [10, 50] and die shortly after birth due to respiratory failure, most likely due to cartilage defects of the rib cage. A human disorder, dyssegmental dysplasia, Silverman-Handmaker type, was identified to be a functional null mutation of perlecan and also showed similar skeletal dysplasia to the knockout mouse [51]. Skeletal defects observed in perlecan-null animals arise largely in the growth plates of the long bones. In normal long-bone growth plates, chondrocytes are arranged into zones of resting, proliferating and maturing (hypertrophic) cells. Growth plates that are deficient in perlecan are severely disrupted in the proliferative and hypertrophic zones.

What is the role of perlecan HS in chondrogenesis?

HS is required in the growth plate for normal development, as demonstrated by mouse knockout studies involving the genes that code for exostosin 1 and 2 (EXT1 and 2), glycosyltransferases required for the assembly of HS. Although EXT1 and 2 homozygous mutant mice die at E6, before the onset of cartilage formation, mice heterozygous for EXT1 show increased chondrocyte proliferation and delayed hypertrophic differentiation [52], and EXT2 heterozygous mice have multiple abnormalities in cartilage differentiation, including disorganization of chondrocytes in long bones [53]. In addition, human defects in the genes encoding EXTs also result in exostoses, ectopic bone growths, whose growth plate is severely disorganized with no HS and reduced and abnormal perlecan distribution [54]. Interestingly, these growth plates resemble those of perlecan null mice.

Perlecan isolated from articular cartilage and chondrocyte cultures is substituted with HS as well as CS chains [55]. HS modification is further supported *in vivo* by the significant levels of HS distributed pericellularly at sites of perlecan deposition in the growth plate [56, 57]. There is currently no evidence that chondrocyte perlecan HS chains are involved in chondrogenesis. However, the following lines of evidence suggest that these chains may be required for the chondrogenic activity attributed to perlecan.

French et al. [58] showed that exogenous addition of domain I of perlecan decorated with either HS or a mix of HS and CS provided a sufficient signal to trigger C3H10T1/2 cells to enter a chondrogenic differentiation pathway. GAG substitution was essential to this activity as neither HS-deficient domain I or any of the recombinant perlecan domains triggered chondrogenesis in these cells. Interestingly, other sources of heparin, HS or CS were also unable to induce chondrogenesis, which suggests that this activity is unique to perlecan-associated GAG.

Given these *in vitro* data, it is surprising that the transgenic mouse deficient in HS-modified perlecan does not exhibit any gross cartilage abnormalities. However, to date there has been no detailed analysis of cartilage structures in these animals. Furthermore, it is possible that domain V of perlecan, which can carry HS chains under some circumstances [12, 59], may compensate for the lack of domain I HS [60]. In support of this, perlecan isolated from the kidneys of these same perlecan-HS-deficient mice was recently shown to be substituted with either HS or CS [61]. The site of GAG attachment was not determined but is likely to be domain V. The lack of a cartilage phenotype could also be a result of redundancy in the functions of HSPGs, as has been observed for the *Drosophila* glypicans Dally and Dally-like. Another possibility is that the activity of perlecan in cartilage is mediated through the protein core rather than the HS chains. This is discussed later.

If the HS chains of perlecan are involved in chondrogenesis their activity may be similar to that of perlecan in the vasculature. Indeed, like endothelial perlecan, perlecan derived from chondrocyte cultures has been shown to interact with the chondrogenic growth factors FGF2 [62] and FGF9 [S. M. Knox and J. M. Whitelock, unpublished data] in an HS-dependent manner. Therefore, similar to angiogenesis, perlecan may influence the activity of key signaling molecules such as FGFs that regulate chondrocyte cell function.

Support for a role of perlecan in the regulation of FGF activity in chondrogenesis comes from the finding that chondrodysplasia observed in perlecan-deficient mice and humans resembled that of FGFR3 gain-of-function mutations. FGFR3 is a negative regulator of skeletal bone growth. Both humans and mice with FGFR3 gain-of-function mutations show an achondroplastic phenotype with inhibition of chondrocyte proliferation at the growth plate, premature differentiation and early cessation of bone growth. It has been postulated by a number of groups that perlecan may regulate the activity of FGFR3 in the growth plate by binding its ligands through its HS chains. Of the 23 FGF family members, FGF2 and FGF18 are thought to be the most likely candidates for controlling bone growth through FGFR3 [63, 64]. FGF18-null mice display a similar phenotype to the FGFR3-null mouse, exhibiting enhanced endochondral bone growth, expansion of their growth plate, and increased chondrocyte proliferation. FGF2-null mice do not show an abnormal skeletal phenotype [31], possibly due to redundancy in the FGF family; however, transgenic mice over-expressing FGF2 or treatment of mouse limb explant cultures with recombinant FGF2 results in severe limb shortening [65, 66]. FGF2 has also been shown to strongly induce the

synthesis of a number of chondrocyte proteins including MMP1 and MMP3, and TIMP-1 [67] and suggests that FGF2 could have a role in remodeling damaged tissue. In addition, FGF2 and FGFR3 have overlapping expression patterns: FGF2 and FGFR3 protein localize predominantly within the proliferating zone. FGF18 mRNA and protein are found in the perichondrium, suggesting a possible paracrine signal to the growth plate [68]. This raises the question of how FGF18 is transported to FGFR3-expressing cells. Given the importance of HSPGs in morphogen gradient formation and that HS binds and activates FGF18 [69], it is possible that perlecan HS chains may also be involved in the diffusion of FGF18 towards FGFR3-expressing chondrocytes.

Interestingly, examination of FGFR3 protein in rib cartilage showed that the transmembrane receptor underwent proteolytic cleavage towards the hypertrophic zone to produce a gradient of an extracellular domain fragment of FGFR3. This fragment showed greatest deposition within the ECM of hypertrophic chondrocytes [70]. Perlecan can bind FGFR3c *in vitro* [25] and may create an extracellular domain FGFR3 gradient, which could then control the availability of FGF.

As the epiphyseal growth plate develops, FGFR3 disappears and FGFR1 expression is upregulated in prehypertrophic and hypertrophic chondrocytes, suggesting a role for FGFR1 in the regulation of cell survival and differentiation, and possibly cell death [64]. Human chondrodysplasias have been mapped to mutations in FGFR1 (and FGFR2), where the majority of syndromes involve cranial disorders. Since perlecan-deficient mice show both skeletal and cranial abnormalities, perlecan may regulate the different forms of FGF receptors. The expression patterns of perlecan and FGFR1 overlap significantly, and similar to perlecan-deficient mice, conditional inactivation of FGFR1 in mice resulted in shortened limbs [71, 72]. It should be noted, however, that the major phenotype of abnormal autopod formation is not seen in perlecan-null mice, although a detailed analysis of the cartilage was not performed in these studies. A recent *in vitro* study by Govindraj et al. [62] suggested that growth plate perlecan may function to sequester FGF2 away from its high-affinity receptor, presumably FGFR1, thereby preventing its activation. Therefore, similar to FGFR3, perlecan may negatively regulate FGFR1 by sequestering its ligand.

As stated above, expression of perlecan is greatest in the hypertrophic regions, and these are also the sites of the highest expression of the heparin-binding bone morphogenic proteins (BMP)-2, -4 and -6. Binding to HSPGs has been implicated in the signaling of BMPs [73], restricting BMP-4 diffusion [74] and is thought to facilitate internalization and/or lysosomal targeting of mature BMP-4 [75]. Although BMP function in chondrocytes is contentious, a recent study using a rat model over-expressing constitutively active BMP receptor 1a in cartilage showed that BMP activity stimulates chondrocytic differentiation and maturation towards hypertrophy [76]. Gomes and coworkers [77, 78] showed that mouse perlecan and recombinant HS-modified domain I of perlecan induced chondrogenic nodule formation of 10T1/2 cells and stimulated BMP2-mediated chondrogenic maturation [78]. Recently, Fisher et al. [79] demonstrated that exogenous HS or heparinase treatment dramatically enhance the ability of BMP2 to stimulate chondrogenesis and cartilage-specific gene expression in limb mesenchymal cells in micromass culture. The authors suggest that this activity is due to the HS chains of perlecan since surface-bound HS, most likely from syndecan-3, was demonstrated to interfere with the chondrogenic activity of BMP.

Hypertrophic chondrocytes of the growth plate also produce VEGF, connective tissue growth factor (CTGF) and MMPs including MMP-2, -3, -7, and -13 [80–83]. Perlecan has been shown to co-localize pericellularly with CTGF and interact with it *in vitro* [84]. This interaction is most likely through the HS chains, as heparinase treatment of cells resulted in release of CTGF [84]. Similar to perlecan, CTGF is expressed highly in vascular tissues and maturing chondrocytes and is believed to be a regulator of both chondrogenesis and angiogenesis during skeletal development [85] by a mechanism that may involve the binding and inhibition of VEGF165 [86]. VEGF is essential for growth plate angiogenesis and subsequent bone formation [87]. Complex formation between VEGF165 and CTGF interrupts binding of VEGF165 to its major receptor VEGFR-2, and thus inhibits VEGF angiogenic activity [86]. Proteolysis of the VEGF165/CTGF complex by MMP-1/-3, -7 or -13, which are upregulated during cartilage remodeling, was shown to degrade CTGF and result in release of active VEGF [88]. Indeed, MMP-13-deficient animals have delayed vascularisation of the ossification center [89] and this is thought to be due to decreased availability of VEGF to interact with its receptors on endothelial cells, osteoclasts and osteoblasts. This mechanism is also supported by the finding that infusion of dimerized soluble receptor Flt-1 (VEGF receptor 1) reversibly delayed bone formation in the growth plate [87]. It should also be noted that perlecan is cleaved by MMP-3 [36], and this may present a mechanism whereby the inactive CTGF/VEGF complex is able to diffuse within the growth plate.

Preceding vessel invasion of the primary ossification center within developing bones, there is recruitment of vessels to the surrounding perichondrium. At the time of this perichondrial recruitment, VEGF is expressed by the perichondrial cells and has been suggested to be involved in the recruitment of blood vessels to the perichondrium [reviewed in ref. 90]. Interestingly, both perlecan and CTGF have also been shown to be expressed in the perichondrial vessels [91, 92] and may therefore control this initial invasion in a mechanism similar to that described

above. Therefore, perlecan HS chains may manipulate the activity of VEGF165 indirectly through binding CTGF to control vascular invasion and subsequent bone formation in the growth plate.

What is the role of the protein core in chondrogenesis?

Similar to that for angiogenesis, there has been very little research on the function of the perlecan protein core in cartilage development. However, like its postulated role in maintaining the structural integrity of blood vessel basement membranes, it is also likely that in normal growth plate cartilage, perlecan strengthens the ECM structure through matrix protein interactions. This is supported by the achondroplastic phenotypes observed in human skeletal disorders linked to mutations in the perlecan core protein. One such disorder is Schwartz-Jampel syndrome (SJS), which is characterized by chondrodystrophic myotonia. SJS has been mapped to the missense and splicing mutations resulting in versions of perlecan either with partial loss of domain IV and complete loss of domain V, complete loss of domain V only, or molecules defective in disulfide bond formation in domain III [93, 94]. There has only been a single report on cartilage from SJS patients and this indicated poor chondrocyte columnar organization in epiphyseal cartilage of one patient with SJS [95]. However, this disorder is also characterized by short stature, osteochondrodysplasia, myotonia and skeletal abnormalities and is therefore indicative of a role for domain V of perlecan in cartilage development as well as muscle and skeletal development. Domain V of perlecan was found to bind fibulin-2, laminin-nidogen complex, nidogen and two nidogen fragments, progranulin [12, 19, 47, 96] and, more recently, extracellular matrix protein 1 (ECM1) [97]. ECM1 has been shown to regulate endochondral bone formation in an organ culture model [98], as well as stimulate the proliferation of endothelial cells, and induce angiogenesis. Its expression also partially overlaps with perlecan. However, ECM1 mutations in humans result in skin rather than skeletal abnormalities, which suggests other roles for perlecan/ECM1 interactions.

Perlecan can also interact with WARP (von Willebrand factor A-domain related protein). WARP is a novel member of the von Willebrand factor A-domain superfamily of ECM proteins and has been shown to co-localize with perlecan in the chondrocyte pericellular microenvironment [57]. *In vitro* studies suggest that it binds the protein core via domain III as well as its HS chains [57]. One hypothesis is that this interaction in articular cartilage may contribute to the assembly and/or maintenance of 'permanent' cartilage structures during development and in mature cartilages; however, the function of WARP remains to be determined.

Recently Govindraj et al. [62] identified a smaller-molecular-weight form of perlecan from bovine growth plate chondrocyte cultures that did not contain HS and was localized to the cell layer. This smaller perlecan has also been observed in human cartilage extracts [J. Melrose, P. Roughley, S. Knox, S. Smith, J. Whitelock, unpublished data]. To date, this is the only variant form of perlecan protein core identified, although an alternatively spliced species of perlecan mRNA was discovered in mice [99]. Whether this form is a result of alternative splicing or proteolytic processing in the cartilage is unknown; however, intensive investigations by Iozzo et al. [100] failed to find alternative splice variants in human perlecan. Such variation in the structural composition of the perlecan protein core will differentially regulate its activity in this tissue. For example, the absence of HS chains prevents perlecan from interacting with HS-binding chondrogenic growth factors such as FGF2 [62] precluding it from modulating FGF2 signaling.

Conclusion

It is surprising, given the critical need for perlecan in normal chondrogenesis and bone development, that almost nothing is known about the mechanism of its action in the generation of this complex multifunctional tissue. It is tempting to speculate that it has a critical role in the patterning of morphogens and mutagens in the developing tissue through regulating the concentration of matrix-bound versus cell-associated (bound to glypicans and syndecans) signaling molecules. Importantly, the ability to establish these concentration gradients is dependent on the glycosylation status of the perlecan molecule. If the glycosaminoglycan structures change as a cell differentiates, these changes will have a downstream effect on the type and distribution of growth factors in the local environment of the cell. This may provide a developmental mechanism to control the various stages of cell differentiation and proliferation during organogenesis.

Thus perlecan regulates and is regulated by cells through a basic mechanism involving the binding of proteins via the protein core and/or the glycosaminoglycan chains. Perlecan functional diversity is due to these interactions being a product of the makeup of the extracellular environment, whose complexity may be far-reaching and solely dependent on the status of the cell. As such, perlecan may be a bridge that provides one of the critical links between cell behavior and organogenesis.

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