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Role of perlecan in skeletal development and diseases

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Perlecan, a large heparan sulfate proteoglycan (HSPG), is present in the basement membrane and other extracellular matrices. Its protein core is 400 kDa in size and consists of five distinct structural domains. A number of *in vitro* studies suggest multiple functions of perlecan in cell growth and differentiation and tissue organization. Recent studies with gene knockout mice and human diseases revealed critical *in vivo* roles of perlecan in cartilage development and neuromuscular junction activity.
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Structure and expression

Proteoglycans consist of a core protein containing one or more glycosaminoglycan (GAG) chains. Most proteoglycans have either chondroitin sulfate or heparan sulfate chains or both. Chondroitin sulfate and heparan sulfate are repeating disaccharides of an amino sugar and an uronic acid. The amino sugar is N-acetylgalactosamine in chondroitin sulfate and N-acetylglucosamine in heparan sulfate. The biosynthesis of chondroitin and heparan sulfate is dependent upon a sequence of prior events: transcription of mRNA for a proteoglycan core protein, translation of the mRNA into a precursor protein and assembly of a chain initiation site on the precursor protein. This latter event involves the addition of xylose to certain serine residues on the precursor protein followed by the sequential addition of two galactoses and a glucuronic acid to complete the tetrasaccharide linkage. Linkage region addition is favored on those serines in the precursor protein that are followed by a glycine [1,2]. The synthesis of the GAG then begins with the addition of N-acetylglucosamine, for heparan sulfate, or N-acetylgalactosamine for chondroitin sulfate [3]. The selective addition of N-acetylglucosamine to initiate a heparan sulfate chain is determined by signals in the precursor protein such as the presence of an adjacent cluster of acidic amino acids [4–6]. (Without such signals, the addition of N-acetylgalactosamine would otherwise occur, and a chondroitin sulfate chain would be initiated at that site.) Synthesis of the heparan sulfate chain then occurs by the polymerization of alternating glucuronic acid and N-acetylglucosamine residues by two glycosyltransferases specific for heparan sulfate synthesis [7,8]. Modifying enzymes then introduce sulfate groups at various positions on the growing chain, and some of the glucuronic acid residues are converted to iduronic acid [9].

There are many different genes and gene families for core proteins of heparan sulfate proteoglycans. These include the four members of the syndecan family, the six members of the glypican family, perlecan, agrin and collagen XVIII [9–11]. The syndecan core proteins have a transmembrane segment. The glypicans have a site on the C-terminus of their core proteins that is involved in the formation of a glycosyl phosphatidyl inositol linkage to the plasma membrane. Consequently, both these gene families are considered to be "cell surface proteoglycans." Perlecan, agrin, and collagen XVIII lack these core protein determinants and are thought to be "matrix proteoglycans."

Perlecan is a heparan sulfate-containing proteoglycan that was first discovered in a murine tumor cell line and was shown to be present in all native basement membranes [12]. Subsequent biochemical characterization of murine perlecan showed it contained an estimated 400-kDa core protein with 2–3 heparan sulfate side chains at the N-terminus and one chondroitin sulfate chain at the C-terminus [13,14]. The amino acid sequence of murine perlecan deduced from cDNA cloning [15,16]

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indicated perlecan's 369-kDa core protein consisted of five distinct domains: domain I is a unique N-terminal sequence that was later shown to contain the sites for heparan sulfate attachment [4]; domain II is similar to the cholesterol binding region of the low density lipoprotein (LDL) receptor; domain III is similar to the short arm of the laminin A chain; domain IV is the largest domain and contains 14 repeats of IgG-like motifs similar to those in neural cell adhesion molecule (N-CAM); and domain V is similar to G subdomains of the laminin chain interrupted by EGF-like motifs. Perlecan's name is derived from its rotary shadowed image in the electron microscope as a string of pearls [16–19]. Perlecan is expressed in multiple forms. The structure of human perlecan is essentially identical to murine perlecan except that domain IV contains 21 repeats of the IgG-like motifs, which increases the core protein to 466 kDa [20]. This longer form of perlecan was shown to be expressed in mice as an alternatively spliced form [21]. A homologue of perlecan is also present in the basement membranes of *C. elegans* [22,23] and *Drosophila* [24]. Mutations in the alternatively spliced perlecan gene in *C. elegans* produce defective muscle development and cause paralysis [22,23]. Perlecan may contain only heparan sulfate chains [12] or both heparan and chondroitin sulfate chains [25]. The size of the GAG chains also varies according to tissue and cell type.

In addition to its presence in all basement membranes, perlecan is also present in the extracellular matrix of the growth plate and in articular cartilage, where it contains both heparan sulfate and chondroitin sulfate chains [25,26]. Interestingly, the greatest deposition of perlecan during embryonic development was found to be in cartilage undergoing endochondral ossification in the growth plate during long bone development [27]. Especially strong expression of perlecan is observed in the prehypertrophic and hypertrophic zones [28]. Although perlecan is considered a "matrix proteoglycan," immunostaining of cartilage sections with antisera to perlecan showed that it is localized at the pericellular space of chondrocytes and in the matrix between chondrocytes [26,28].

The human gene for perlecan (*HSPG2*) is larger than 120 kb in size. The gene structure was analyzed, and the number of exons was determined to be 94 [29]. However, the recent human chromosome 1 working draft sequence revealed that the perlecan gene consists of 97 exons (GenBank accession number NT_004576) [30,31]. The exon-intron organization shows a good correlation with the corresponding domains of the homologous genes. This suggests that the perlecan gene evolved by exon shuffling and gene duplication of a common ancestor. The promoter of the perlecan gene lacks TATA or CATT boxes, which are characteristic for house-keeping genes. Some regulatory regions have been identified, e.g., transforming growth factor-β (TGF-β) responsiveness [32].

Molecular interactions

Perlecan binds a number of extracellular matrix molecules, cell surface receptors, and growth factors, suggesting that perlecan acts as a glue to stabilize matrix organization and cell-matrix interaction and to modulate growth factor signaling for cell proliferation and differentiation [33,34]. Perlecan has been shown to bind basement membrane proteins including laminin, collagen IV, and nidogen/entactin [35]. It also binds other extracellular matrix including fibulin and fibronectin. Binding domains of perlecan for these interactions have been identified using recombinant proteins. Domain V interacts with cell surface receptors such as integrin $\beta 1$ and α-dystroglycan [36,37]. Many growth factors such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) bind the heparan sulfate chains and the protein core of perlecan [38–40]. It has been shown that perlecan is a low-affinity binding molecule for β-FGF and promotes mitogenesis and angiogenesis [41].

Gene knockout mice

Gene knockouts in mice have been useful in identifying the essential role of genes in development and tissue organization. The perlecan-null mutation in mice was independently accomplished by two research groups [28,42] and they reported similar perinatal lethal chondrodysplasia. Some of the *perlecan* $-/-$ mice died at embryonic day 10.5 with defective cephalic and cardiac development. Electron microscopy indicates that this may be due to altered basement membrane formation in the absence of perlecan. The remaining *perlecan* $-/-$ mice go on to develop skeletal dysplasia characterized by shortened long bones and craniofacial abnormalities and then die shortly after birth. Histological evaluation of the *perlecan* $-/-$ mice revealed a disorganized growth plate with reduced chondrocyte proliferation and differentiation and defective endochondral ossification [28,42]. Severe disruption of the matrix structure in the hypertrophic zone was observed especially late stages of development. Initially, no significant cartilage abnormalities were found prior to embryonic day 13.5, at which time endochondral ossification starts and thereafter disruption of the matrix became obvious and progressively severe. This accounted for the shortened long bones. Interestingly, abnormalities of phalange cartilage were less severe. Thus, perlecan is essential for normal cartilage development in mice.

The obligatory role that perlecan plays in cartilage development is not known. Perlecan isolated from non-cartilage cells and tissues has been shown to bind FGFs and growth factor-binding proteins by interactions with perlecan's core protein and its GAG chains [43–47]. FGFs are a family of proteins that act as intercellular signals in a wide variety of developmental processes [48], including limb development [49]. Transgenic mice over-expressing FGF-2 [50] and FGF-9 [51] exhibit defective growth plate development. More recently, it was shown using gene knockout mice that FGF-18 plays a critical role in endochondral ossification by increasing chondrocyte proliferation and differentiation and Indian hedgehog (Ihh) signaling [52]. The growth plate phenotype of the *Fgf18* knockout mice is similar to *Fgfr3* knockout mice, which are characterized by extended growth plates due to increased proliferating and

hypertrophic chondrocyte zones. Opposite cartilage abnormalities, i.e., short growth plate due to reduced proliferation and differentiation of chondrocytes, were observed in the most common human lethal chondrodysplasia, thanatophoric dysplasia type I, which is caused by activating mutations in *Fgfr3* [53]. FGF-R3c is a chondrocyte-specific receptor that suppresses expression of Ihh, which promotes chondrocyte proliferation. Ihh [54] has been shown to bind to heparin, a structural homologue of heparan sulfate, and to contain an attached cholesterol moiety [55,56]. While perlecan has not yet been demonstrated to bind to Ihh, perlecan does have heparan sulfate chains attached to its domain I [4], and domain II of its core protein has structural similarity to the region of the LDL receptor that binds cholesterol. Thus, perlecan could bind to both the cholesterol moiety and the heparan binding site of Ihh and thereby provide a very specific interaction. We and others have proposed [28,57] that perlecan could be a participant in the signaling pathways that regulate chondrocyte proliferation and hypertrophy. The heparan sulfate chains on perlecan may modulate the activity of an FGF receptor (FGF-R3) in growth plate chondrocytes by binding FGF [58]. Alternatively, or additionally, perlecan may modulate parathyroid hormone related peptide expression by regulating the diffusion of Ihh in the growth plate [59]. Another function of perlecan in cartilage development could be to stabilize matrix and cell-matrix interactions. Without perlecan, the matrix structure of chondrocytes is disrupted, and without a stable matrix cells are not able to differentiate.

Dyssegmental dysplasia, Silverman-Handmaker type

Similarities of the skeletal abnormalities of perlecan knockout mice led to identification of a human disorder, Dyssegmental dysplasia, Silverman-Handmaker type. This is a rare lethal autosomal recessive skeletal dysplasia characterized by anysospondyly and micromelia. Three patients were identified for perlecan mutations. A homozygous 89-bp duplication mutation was found in exon 36 of the perlecan gene in a pair of siblings with this dysplasia [60]. Heterozygous point mutations were identified at the donor site of exon 54 and at the middle of exon 73 in a third unrelated patient, causing exon skipping. These mutations are predicted to produce premature termination codons. Immunostaining and Western blotting revealed that truncated proteins were synthesized but not secreted. Thus, this dysplasia is caused by functional null mutations of the perlecan gene similar to the gene knockout mice [61].

Neuromuscular junction of knockout mice

Perlecan is present in muscle basement membranes and is enriched at the neuromuscular junction [62,63]. This junction forms a highly specialized structure where a unique set of molecules such as acetylcholinesterase, acetylcholine receptors, agrin, dystroglycans, rapsyn, and utrophin are clustered. In skeletal muscles, acetylcholine acts as a neurotransmitter. It binds the acetylcholine receptor on the muscle cell surface, activates Na channels, and induces muscle contraction. The esterase rapidly inactivates this process for muscle relaxation and recycling of acetylcholine. The collagen-tail form of the acetylcholine esterase is preferentially expressed in innervated regions of muscles and is shown to bind perlecan *in vitro* [64]. In the perlecan knockout mice, muscle development and differentiation appear to be normal and the nerve terminals are normally formed. Clustering molecules are present at the neuromuscular junction of the mutant mouse muscles. However, the acetylcholine esterase is completely absent at the neuromuscular junctions, although it is synthesized normally [65]. Thus, perlecan is essential for localizing acetylcholine esterase at the neuromuscular junction.

Schwartz-Jampel syndrome

Schwartz-Jampel syndrome was initially identified for perlecan mutations by positional cloning and gene linkage [30]. This syndrome is characterized by a unique combination of myotonia and chondrodysplasia, and unlike patients with the dyssegmental dysplasia, patients with Schwartz-Jampel syndrome survive. Characterization of the perlecan gene in these patients showed that it contained homozygous missense and splicing mutations that could result in either truncated perlecan missing part of domain IV and the whole domain V or molecules defective in disulfide bonds in domain III. Recent studies on additional Schwartz-Jampel syndrome patients [31] revealed various types of mutations that resulted in various forms of perlecan. For example, heterozygous mutations produce either truncated perlecan that lacks domain V or significantly reduced levels of wild-type perlecan. A homozygous 7-kb deletion resulted in reduced amounts of nearly full-length perlecan. Unlike the dyssegmental dysplasia syndrome, the Schwartz-Jampel syndrome mutations result in different forms of perlecan in reduced levels that are secreted to the extracellular matrix and are partially functional. These findings indicate an important role of perlecan in neuromuscular function and cartilage formation. Recently, Spanger et al. [66] reported that clinical examinations indicated that other skeletal dysplasias previously identified as chondrodysplasia, (kyphomelic chondrodysplasia, micromelic chondrodysplasia, and Burton disease), could be reclassified. Thus, Schwartz-Jampel phenotype encompasses a wide spectrum.

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