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Perspective Article

Hylonome

Perlecan: an important component of the cartilage pericellular matrix

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Perlecan (Pln) is a large proteoglycan that can bear HS (heparan sulfate) and chondroitin sulfate glycosaminoglycans. Previous studies have demonstrated that Pln can interact with growth factors and cell surfaces either via its constituent glycosaminoglycan chains or core protein. Herein, we summarize studies demonstrating spatially and temporally regulated expression of Pln mRNA and protein in developing and mature cartilage. Mutations either in the Pln gene or in genes involved in glycosaminoglycan assembly result in severe cartilage phenotypes seen in both human syndromes and mouse model systems. *In vitro* studies demonstrate that Pln can trigger chondrogenic differentiation of multipotential mouse CH310T1/2 stem cells as well as maintain the phenotype of adult human chondrocytes. Structural mapping indicates that these activities lie entirely within domain I, a region unique to Pln, and that they require glycosaminoglycans. We also discuss data indicating that Pln cooperates with the key chondrogenic growth factor, BMP-2, to promote expression of hypertrophic chondrocyte markers. Collectively, these studies indicate that Pln is an important component of human cartilage and may have useful applications in tissue engineering and cartilage-directed therapeutics.

Abbreviations: BMP, Bone Morphogenetic Protein; DDSH, Dyssegmental Dysplasia Silverman-Handmaker Type; ECM, Extracellular Matrix; HS, Heparan Sulfate; HSPG, Heparan Sulfate Proteoglycan; Pln, Perlecan; SJS, Schwaltz-Jampel Syndrome.

Introduction

Cartlilage development and maintenance involves a complex interplay among transcription factors, growth factors and ECM. While important molecules in each class have been identified, a complete picture is not available, nor is it known how each of these influences modulates the activity of the others. Furthermore, it is not clear how these processes differ in the development and maintenance of articular cartilage and cartilage of endochondral bone. ECM components serve as markers of chondrogenic progression and, more recently, have been recognized to play active roles in cartilage development. In this report, we will focus on studies of the expression and function of Pln, a proteoglycan of cartilage ECM that appears to coordinate aspects of cartilage development through a combination of interactions with chondrocytes as well as chondrocytic growth factors.

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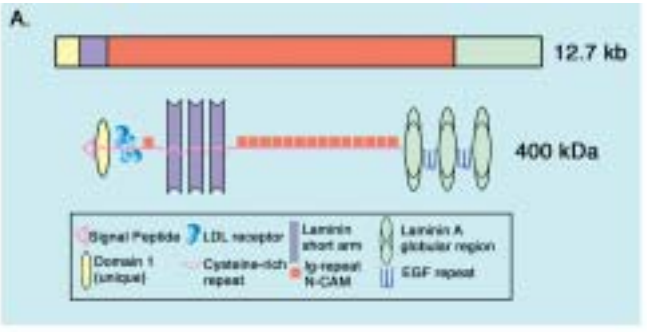
Growth regulators and chondrogenesis

Cartilage formation occurs early during embryonic development and is required for the formation of skeletal structures. *In vitro* and *in vivo* developmental studies designed to identify key molecules expressed in chondrogenic primordia and important in the subsequent formation of skeletal elements and their joints provide insight into the mechanisms underlying the biology of both normal and pathologic adult cartilages. Although the origin of chondroprogenitor cells differs depending on the embryonic location in which they arise, differentiation into mature chondrocytes takes place via a similar multistep cascade of cellular events. First, mesenchymal cells committed to the chondrogenic fate are recruited and their subsequent condensation is followed by frank differentiation into chondrocytes secreting a characteristic surrounding pericellular matrix composed of marker proteins including type II collagen and aggrecan[1]. The maturing chondrocytes are then flattened and form columns proliferating unidirectionally, and are progressively withdrawn from the cell cycle (prehypertrophy) to change their genetic program and, in the case of growth plate, undergo



B.

Comparison of Mouse and Human PLN

Property	Mouse	Human
#amino acids (- signal peptide)	3707	4370
mRNA size	12.7 kb	~13 kb
N-terminal HS attachment sites	3	3
RGD in domain III	Yes	No
Chromosome assignment	4	1

Figure 1: A) Domain structure of Pln showing five major domains, the first of which contains the glycosaminoglycan chains and is unique. B) Comparison of key properties of mouse and human Pln.

hypertrophic growth accompanied by the synthesis of new marker proteins such as type X collagen and alkaline phosphatase. These events require the coordination of both soluble bioactive growth factors and components of the ECM itself[2]. Several human cartilage disorders are due to loss-of-function mutations in specific ECM and cell-surface HSPG genes such as syndromes resulting from mutations in the Pln and glypican-3 genes[3,4]. Gene disruption of Pln and glypican-3 in mice resulted in various skeletal abnormalities analogous[5-7] to the clinical features observed in humans (chondorodystrophias/mandibular hypoplasia). Another syndrome, hereditary multiple exostoses, the most common type of tumor disorder developing primarily in long bones and characterized by inappropriate cartilage growth, results from mutations in the EXT gene family encoding for enzymes involved in HS chain assembly on proteoglycan core proteins[8,9]. Chondrogenesis is a complex process involving the coordinated action of many players including growth factors, their receptors, and molecules controlling their bioactivity both extracellularly and intracellularly.

Many chondrogenic growth factors share the property of selectively binding to HSPGs present at the cell surface and in the ECM. In several cases, these high affinity interactions lead to localized increases in soluble HS-binding growth factors to concentrations or conformations optimal for interaction with signaling receptors[10]. Many studies describe the expression and involvement of HS-binding growth factors during the steps leading to chondrocyte differentiation[11-13]. Notable examples include bone morphogenetic proteins (BMPs), fibroblast growth factor-2, and heparin-binding growth-associated molecule-pleiotrophin / midkine family members.

Pln structure and biology

Several groups, including our own, have described a striking accumulation of the basement membrane HSPG, Pln, in cartilage of developing and adult animals in various species[14-16]. In addition, the mouse and human Pln genes have been mapped to chromosome 4, and the telomeric region of chromosome 1, respectively[17,18]. Pln is a large, multidomain protein of 400-470 kDa encoded by an approximately 12 kb mRNA (Figure 1). The protein core is composed of 5 major domains with sequence similarities to other ECM and cell surface proteins and is found in species as diverse as nematodes and humans, implicating a fundamental biological role[19]. In addition, cDNA cloning and expression of Pln domains in mammalian cell lines have provided valuable tools for the study of the potentially distinct functions of individual Pln domains[20-23]. Three potential glycosaminoglycan attachment sites occur near the amino terminus. In most cases, the glycosaminoglycans are of the HS variety, although chondroitin sulfate may sometimes substitute for HS at one or more of these sites[14]. The amino terminal, HS-bearing domain I of Pln is unique in sequence while the other four domains share extensive sequence homologies with other cell surface and ECM proteins. The gene structure of human Pln and part of its promoter region have been described[24]. Limited information is available on the transcriptional regulation of the Pln gene[25,26]. There are no studies that specifically address transcriptional regulation of Pln gene expression in chondrocytes. It has been suggested that Pln accumulation in cartilage primarily reflects enhanced Pln protein stability[15,16]. If this is true, it seems unlikely that cartilage-specific transcriptional regulation is a key feature in this system.

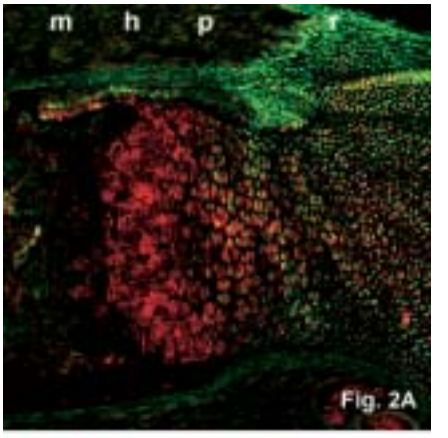
The large, complex nature of Pln suggests that this proteoglycan interacts with multiple cell surface and ECM proteins. Several growth factors as well as other soluble proteins of the cell surface and extracellular matrix bind to the HS chains of Pln[27,28]. In some cases, secreted factors appear to bind to the Pln core protein[29,30]. On the other hand, relatively little is known about the cell surface proteins that mediate interactions with Pln. Several studies suggest that integrins, particularly those of the β1 family, mediate Pln binding to cells[22,31,32]. Mouse Pln contains a single RGD sequence in domain III; however, this site is not conserved in the human protein and the recombinant domain III fails to support cell attachment or bind to α5β1 or αvβ3 integrins[20]. Therefore, the function of the Pln RGD sequence is questionable. In the context of chondrogenesis, many studies demonstrate that β1 integrin-containing complexes appear in cartilage of

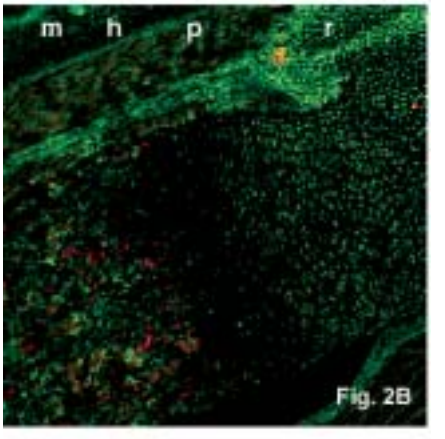
developing tissues and in adult animals[33-35]. Furthermore, β1 integrin-specific antibodies inhibit chondrogenesis *in vitro*[36,37]. Thus, β1 integrins are appropriately expressed to support Pln-dependent interactions with chondrocytes. Chondrocytic cell lines have been shown to attach to Pln-coated matrices[14]. Nonetheless, until recently[16], no formal demonstration of Pln binding to chondrocytes or chondrocytic precursor cells has been made and no information on Pln interactions with β1 integrins on chondrocyte cell surfaces is available. Binding of certain proteins to cell surface receptors requires both protein-protein and protein-HS interactions[38]. Consequently, both the constituent HS chains and core protein of Pln may be involved in interactions with cell surface components.

Pln and HSPG functions in cartilage development

The cartilage ECM contains a variety of proteoglycans, the majority of which carry glycosaminoglycan chains other than HS; however, during the past decade Pln has received increased attention as an important ECM molecule during cartilage development. Recently, Pln was determined to be the major HSPG of the bovine growth plate[39]. The earliest reports localized Pln to the pericellular matrix of avian, human, and murine articular and growth plate cartilage cells[14,19]. Developmental studies of Pln expression in cartilage during murine embryogenesis have been reported by two independent groups[15,16]. Together, their findings demonstrated that Pln expression is pronounced within cartilaginous tissues especially those undergoing endochondrial ossification (Figure 2). In these structures Pln is first detected at day 10.5 and accumulates primarily at the hypertrophic zone of the growth plate, throughout the remaining developmental stages. Immunostaining with antibodies to collagen type II, a hallmark of chondrocyte differentiation, is apparent at a time when Pln is only faintly detected[16], suggesting that while Pln expression is a component of the chondrogenic program, it does not initiate this process.

Pln gene knockout in mice produces a lethal embryonic phenotype, at least for the majority of mice[5,6]. Curiously, the small percent of Pln nulls that survive until birth present severe skeletal defects and chondrodysplasias. In the developing long bones of these animals, normal organization within the proliferative and hypertrophic zones of the growth plate is lost[5,6]. Combined, these studies suggest that Pln plays an important organizational role in normal growth plate development. Recently, a comparison of skeletal abnormalities in human patients to those in Pln-null mice[40,41] identified mutations in the Pln gene that give rise to two classes of skeletal disorders: the relatively mild Schwartz-Jampel syndrome (SJS) and the severe neonatal lethal dyssegmental dysplasia, Silverman-Handmaker type (DDSH). Pln is not detected in the cartilage of patients with DDSH[40] ; however, mutant Pln molecules or reduced amounts of wild type Pln are localized in the pericellular matrix of cartilage tissues





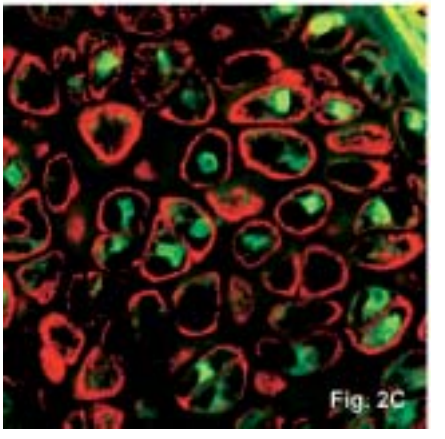


Figure 2: Pln in cartilage. Pln (red staining) is expressed throughout murine metatarsal growth plate (Panel A) and cranial cartilage (Panel C). Non-immune control staining is shown for growth plate cartilage in Panel B. Green staining is for a nuclear dye, syto 13. In panels A and B, a region containing resting (r), proliferative (p), hypertrophic (h) chondrocytes as well as the metaphysis (m) is shown. In panel C, note the intense staining in the pericellular matrix.

from patients with SJS[41]. The literature contains only one report regarding the histology of cartilage from patients with SJS. Similar to Pln null mice, this SJS patient presents abnormal chondrocyte columnar organization within epiphyseal cartilage[42]. Collectively, these findings suggest that Pln has an important role in cartilage and subsequent bone develop-

ment in animals, and begin to define the molecular basis for the phenotypic differences associated with alterations in normal Pln expression. However, the mechanistic actions of Pln in cartilage development, maturation and turnover remain largely elusive.

Previous studies with cultures of chick limb bud mesenchymal cells have suggested a role for HS in formation and growth of chondrogenic regions. This effect was deemed dependent on HS chain structure, size, and charge. Chondroitin sulfate, lacking L-iduronic acid, and less sulfated than HS derivatives, had little activity. The presence of HS or heparin was proposed to promote the growth of the aggregates by assisting in the formation of tighter aggregates, thereby detaching the cells from matrix[43]. More recent investigations employing the multipotential mouse embryonic fibroblast cell line C3H10T1/2, suggest that while these cells bind well to a variety of purified ECM components, Pln, from two different sources (EHS tumor, WiDr), uniquely stimulates conversion to a chondrogenic phenotype *in vitro*[2,16,44-47]. Characterized by rapid cell condensation and aggregate formation, the phenotypic response of C3H10T1/2 cells plated on a Pln matrix also leads to the accumulation of alcian blue staining material, and expression of early stage chondrogenic marker proteins (aggrecan, type II collagen[44]); however, unless cultures are supplemented with rhBMP-2(2), C3H10T1/2 cell aggregates fail to morphologically resemble or express markers of mature chondrocytes (i.e., type X collagen). These observations suggest that Pln, alone, promotes the early stages of chondrogenic conversion *in vitro*, but is not sufficient to drive subsequent developmental stages.

Studies designed to determine which Pln domain(s) promoted C3H10T1/2 cell aggregation and chondrogenic differentiation[44], demonstrate that recombinant Pln domain I, but not recombinant Pln domains II-V are responsible for C3H10T1/2 aggregation and chondrogenic differentiation. The response of C3H10T1/2 cells plated on recombinant Pln domain I-coated surfaces is identical to the response generated when the same cells are plated on intact Pln (EHS tumor or WiDr derived). Interestingly, the "domain mapping" investigation also identified an important role for glycosaminoglycan chains. In short, enzymatic removal of glycosaminoglycans or genetic removal of glycosaminoglycan attachment sites on intact Pln or recombinant Pln domain I abolishes chondrogenic activity[44]. In this regard the observation that Pln maintains the differentiated chondrogenic phenotype of both normal human and exostoses chondrocytes *in vitro*[8,16] is of particular interest. The EXT gene family code for enzymes that regulate HS chain assembly on proteoglycans. Mutations in this gene family are the underlying genetic defect in hereditary multiple exostoses in humans[9]. Most recently, we have come to learn that growth plates of exostoses patients are devoid of HS, and show an abnormal Pln distribution[48].

Conclusions

Pln is highly expressed in both developing and adult cartilage in a temporally and spatially restricted pattern. The ability of this proteoglycan to bind key growth factors as well as cell surface components indicates that it helps coordinate proper cartilage growth and development. Consistent with this idea are observations in Pln null mice and human mutations in genes encoding Pln or HS assembly enzymes that result in cartilage abnormalities. Further work should reveal the precise molecular interactions that occur between Pln and other molecular components of cartilage. Furthermore, the ability of Pln to stimulate cartilage differentiation in mesenchymal precursors indicates that this molecule may have promise in cartilage tissue engineering applications.

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