RICE UNIVERSITY

An Investigation of the Biophysical and Biochemical Properties of Perlecan/HSPG2: 
Implications for Bone Mechanotransduction and Endochondral Ossification

by

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A DISSERTATION SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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HOUSTON, TEXAS
May 2016
Abstract

An Investigation of the Biophysical and Biochemical Properties of Perlecan/HSPG2: Implications for Bone Mechanotransduction and Endochondral Ossification

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Jerahme Ryan Martinez

Perlecan, also known as heparan sulfate proteoglycan 2 (HSPG2), is a very large secreted proteoglycan ubiquitously expressed in all basement membranes and in the territorial matrix of skeletal tissues. Perlecan is particularly important for the formation and stabilization of tissue layers and its absence is catastrophic for bone and cartilage development. In mineralized bone, perlecan is a key component of pericellular matrix (PCM) surrounding osteocytic processes that preserve fluid flow throughout bone tissue. In addition, recent findings have coupled perlecan expression, along the osteocyte cell surface, to bone mechano-adaptive response. Perlecan’s mechanical properties, important for maintaining the osteocyte cell-bone matrix interface, were studied for the first time. This work demonstrated perlecan functions as a semi-flexible tether that is capable of withstanding physiological loads imposed on cortical bone. The second part of this study examined perlecan in the context of early precartilage condensation. This study demonstrated a novel sub-domain of perlecan, PLN IV-3, located in its fourth domain modulates cell-matrix interactions that are involved in the chondrogenic process. When presented as part of the substratum, PLN IV-3 suppressed focal adhesion kinase (FAK) phosphorylation and signaling through the mitogen-activated protein kinase (MAPK) cascade. This led to reduced cell migration, increased cell-cell adhesion, and decreased cell proliferation, all of which are hallmarks for mesenchyme condensation and subsequent precartilage formation. I hypothesize that this is a possible mechanism by which perlecan functions to coordinate the migration of mesodermal cells and drive chondrocyte differentiation as seen during development. Together, these studies suggest perlecan possesses multifunctional properties that affect bone health throughout various stages of life.
Dedication

This dissertation is dedicated to my parents; my father Marcos for teaching me hard work is its own reward, and my mother Melissa for giving me the strength to overcome life’s greatest challenges.
Acknowledgments

I would like to express my deepest gratitude to my dissertation advisor, Dr. Mary Cindy Farach-Carson, for all her support and guidance. Her passion for research and learning creates a similar drive in all her students. I will forever be grateful for all she has taught me.

I must thank all the members of the Farach-Carson and Carson lab groups. I was fortunate to work with a great group of researchers. Although every one of them had an impact on my education and in some way or another helped shape this project, I would like to recognize a few individuals who were key to my success. Drs. Curt R. Warren and Brian J. Grindel were my mentors throughout this entire process and taught me many of the techniques I used to complete this work. Dr. Daniel D. Carson was like a second advisor to me and always provided valuable feedback, particularly with research designs and strategies. I would like to thank Drs. Pamela E. Constantinou and Daniel A. Harrington for providing me with all the tools I needed to complete my studies, and for sharing their time and knowledge.

I would like to thank everyone I have collaborated with: Drs. Ching-Hwa Kiang and Sithara S. Wijeratne for introducing me to the world AFM, Dr. Lewis Francis and Ben Morgan for welcoming me to Swansea, Drs. Peter Lwigale and James W. Spurlin who worked with me on the AFM/cornea project, and for AFM training, Dr. Liyun Wang for showing interest in my studies and for the many insightful discussions I have had with all of them.

I would like to thank Rice University’s Alliances for Graduate Education and the Professoriate Program for all of their support.

I would like to thank my committee members, Drs. Ching-Hwa Kiang, Michael Stern, and Yizhi Jane Tao, for all their guidance through this journey.
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EQUATION 2.1 – TANGENT TO TANGENT CORRELATION FUNCTION

$$\langle \cos(\theta) \rangle = \exp(-L / 2L_p)$$

EQUATION 2.2 – EXTENSIBLE WORM-LIKE CHAIN MODEL

$$x = L_c \left[ 1 - \frac{1}{\left( 4\beta L_p F \right)^{1/2}} + \frac{F}{K} \right]$$

EQUATION 2.3 – INEXTENSIBLE WORM-LIKE CHAIN MODEL

$$F(x) = \frac{1}{L_p \beta} \left[ \frac{1}{4(1-x/L_c)^2} + \frac{x}{L_c} - \frac{1}{4} \right]$$
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>c-Fos</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>colony forming unit granulocyte-macrophage</td>
</tr>
<tr>
<td>COJ</td>
<td>chondro-osseous junction</td>
</tr>
<tr>
<td>Col2a1</td>
<td>collagen, type 2, alpha 1</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
</tr>
<tr>
<td>CSF-1</td>
<td>colony stimulating factor-1</td>
</tr>
<tr>
<td>DDSH</td>
<td>dyssegmental dysplasia, Silverman-Handmaker</td>
</tr>
<tr>
<td>Dmp1</td>
<td>dentin matrix protein 1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Fgf</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>Hox</td>
<td>homeobox</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>Hspg2</td>
<td>heparan sulfate proteoglycan 2</td>
</tr>
<tr>
<td>Hz</td>
<td>hypertrophic zone</td>
</tr>
<tr>
<td>Ihh</td>
<td>indian hedgehog</td>
</tr>
<tr>
<td>Jun</td>
<td>jun proto-oncogene</td>
</tr>
<tr>
<td>LCS</td>
<td>lacuna-canalicular system</td>
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<tr>
<td>Mcp-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MEPE</td>
<td>matrix extracellular phosphoglycoprotein</td>
</tr>
<tr>
<td>Mitf</td>
<td>microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>OI</td>
<td>osteogenesis imperfecta</td>
</tr>
<tr>
<td>Opg</td>
<td>osteoprotegerin</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PCM</td>
<td>pericellular space</td>
</tr>
<tr>
<td>Pc</td>
<td>perichondrium</td>
</tr>
<tr>
<td>Po</td>
<td>periosteum</td>
</tr>
<tr>
<td>PHz</td>
<td>prehypertrophic zone</td>
</tr>
<tr>
<td>PLN</td>
<td>perlecan</td>
</tr>
<tr>
<td>Pz</td>
<td>proliferation zone</td>
</tr>
<tr>
<td>Phex</td>
<td>phosphate-regulating neutral endopeptidase on chromosome X</td>
</tr>
<tr>
<td>PU.1</td>
<td>spi-1 proto-oncogene</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>Rank</td>
<td>receptor activator of nuclear factor kappa B</td>
</tr>
<tr>
<td>Rankl</td>
<td>receptor activator of nuclear factor kappa B ligand</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine, glycine, asparagine</td>
</tr>
<tr>
<td>Runx2</td>
<td>runt-related transcription factor 2</td>
</tr>
<tr>
<td>SMFM</td>
<td>single molecule force measurement</td>
</tr>
<tr>
<td>SJS</td>
<td>Schwartz-Jampel syndrome</td>
</tr>
<tr>
<td>Sox9</td>
<td>sex determining region Y-box 9</td>
</tr>
<tr>
<td>Tgf-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Trol</td>
<td>terribly reduced optic lobes</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless</td>
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</table>
Chapter 1: Introduction to the skeletal system

This dissertation focuses on the properties of the extracellular matrix (ECM) that affect bone and cartilage tissue quality and function. Chapter 1 will provide an overview of bone biology and discusses matters relevant to a key proteoglycan found in bone and cartilage, perlecan/HSPG2. Furthermore, the role cartilage plays in bone development and function will be discussed in detail, as this is the template from which all endochondral bone is created.

1.1 Anatomy and physiology of the human skeleton

Bone is hard, rigid, calcified connective tissue that makes up the skeleton. Bone provides the framework to support the body, allows for movement, and protects vital organs. Although often thought of as inert calcified structure, bone is in fact a multifunctional organ that houses the hematopoietic system and is a major storage and homeostatic system for calcium and phosphorus (Bandyopadhyay-Ghosh, 2008). The major components of bone are the specialized bone cells and the mineralized matrix, which consists of inorganic minerals, proteins, and glycoproteins. The composition of the matrix itself is approximately 30% organic material, while the remaining 70% consists of inorganic salts (Lasch and Kneipp, 2008). Collagen, an elastic protein that gives bone tensile strength, is the primary component of the organic matrix (Evans and Vincentelli, 1969). The primary inorganic component is hydroxyapatite, a calcium phosphate mineral, which provides rigidity and compressional strength to bone (Bhowmik et al., 2007; Martin and Brown, 1995). Bone-associated tissues such as cartilage, tendons, ligaments and muscle are critical for a proper formation and function of the skeleton. The tendons and ligaments are tough fibrous connective tissue that connect muscle to bone, and bone to other bone, respectively. Cartilage is a flexible connective tissue that is found in bone joints, the rib cage, the intervertebral discs and
many other tissues. The muscles are the driving force for body movement and also stabilize the bones in place.

1.2 Types of bone
The adult human skeleton is composed of a total of 206 individual bones (Tözeren, 2000). Based on shape, bone can be classified as: long bones, short bone, flat bone, irregular bone or sesamoid bone (Clarke, 2008). A schematic of the human skeleton is shown in figure 1.1 A. It is important to note that not all bone is produced the same way. Details of the two processes (intramembranous vs. endochondral ossification) will be discussed in sections 1.4.1-2.

Classification of long bones includes criteria that they be longer in length than width, contain growth plates at the distal ends, and have a protective articular cartilage covering at the ends. The femur and tibia are examples of long bones and some of the largest of the human body.

Short bones are roughly as wide as they are long. These includes the carpal (wrist) and tarsal (feet) bones, which are important for providing support and stability with relatively little movement. Flat bones are strong and flat as the name suggests. Examples of flat bones include the scapula (shoulder blade), sternum (breast bone), and cranium (skull). Their main function is to protect vital organs of the body and form the base for muscular attachment. Interestingly, flat bones produce the highest numbers of red blood cells. Irregular bones have non-uniform shapes and fail to be defined by the previous classifications. Bone of the vertebrate spinal column is an example of irregular bone with highly specialized function. Sesamoid bones are short and irregular, but are imbedded in a tendon. These bones are usually located over joints, where they protect the tendon. The most well-known sesamoid bone is the patella (kneecap), which sits between the patella tendon and quadriceps tendon.
Figure 1.1 The human skeleton and bone structure. (A) The adult skeleton is composed of a total of 206 bone. Bones are categorized as either long bones, short bones, flat bones, irregular bones or sesamoid bones. A representative structure of an adult long bone is shown in (B). Compact bone forms the hard outer layer of bone. On the outer surface of bone is a thin periosteum coating. Just beneath compact bone is the endosteum, which forms the lining of the medullary cavity. Cancellous bone, or spongy bone, is found at the ends of the bone and along the wall of the medullary cavity. Yellow marrow is found directly at the center of bone and red marrow is found dispersed throughout cancellous tissue. Blood vessels and nerves infiltrate the bone network into the center cavity. Illustrations were adapted from Servier Medical Art by Servier
1.3 Bone Structure

Bone is organized into distinct layers. Lining the outside surface of bone is the periosteum, a dense connective tissue composed of an outer fibrous layer and inner osteogenic layer (Roberts et al., 2015). The outer portion of the periosteum contains fibroblasts, while the inner layer contains a pool of osteoprogenitor cells that are important for bone growth and healing bone fractures (Allen et al., 2004; Einhorn, 1998). Cortical bone makes up the outer compact surface, which creates a dense and smooth exterior (Clarke, 2008). This type of bone accounts for most of the adult bone mass. The surface of bone appears uniformly solid and compact, but it is in fact porous with a hollow inner core. Inside cortical bone there are tiny narrow passages, or canals, filled with nerves and blood vessels. Underneath the cortical layer is the endosteum, which separates the cortical bone from the inner cancellous bone (Einhorn, 1998). The endosteum is a thin vascular membrane of connective tissue. The endosteum forms the medullary cavity where bone marrow is stored. Cancellous bone, or trabecular bone, constitutes the interior area of bone (Clarke, 2008). This spongy and porous tissue provides space for bone marrow, nerves, blood vessels and hematopoietic stem cells, which give rise to red and white blood cells, and platelets (figure 1.1 B).

1.4.0 Osteogenesis: the development of bone

Bone is produced by one of two processes, either intramembranous or endochondral ossification (Bronner, Farach-Carson, & Farach-Carson, Ch.1, 2003; Gilbert, 2000). Intramembranous ossification occurs in the flat bones of the skull and clavicle. Most of the human skeleton is formed by endochondral ossification. In intramembranous ossification, mesenchymal cells differentiate directly into the osteoblast lineage, whereas in endochondral ossification mesenchymal cells differentiate into the chondrocyte lineage first. Although both processes are
initiated by the condensation of undifferentiated mesenchymal stem cells. This section will provide a brief overview of the two processes and highlight key differences between the two.

1.4.1 Intramembranous ossification

The major distinction between the intramembranous ossification and endochondral ossification is that cartilage is not present during the intramembranous ossification. As outlined in figure 1.2 A, the process of intramembranous ossification begins with the condensation of small group of neural crest-derived mesenchymal cells (Jiang et al., 2002). As these cells replicate, a tight dense cluster known as the ossification center develops. Cells within the nidus undergo a series of morphological changes, and develop into osteoblasts. Activation of Cbfa1 and BMP signaling are important for the differentiation of neural crest stem cells to osteoblasts (Ashique et al., 2002; Komori et al., 1997; Otto et al., 1997). In contrast, the Wnt/β-catenin pathway, specifically Wnt16 signaling, appears to oppose osteoblast formation (Jiang et al., 2014). Loss of mesenchymal phenotype becomes apparent and cells began to take on a columnar shape with increased amount of Golgi apparatus and rough endoplasmic reticulum. Osteoblasts at the ossification center become trapped in their calcified bone matrix and differentiate into osteocytes (figure 1.2 B). Blood vessels infiltrate the bone tissue creating a network of trabeculae, or woven bone (figure 1.2 C). Osteoblasts along the periphery of the ossification center remain active and produce a plate of compact bone that surrounds the spongy inner core (figure 1.2 D). Eventually the spongy portion of the bone tissue will become the marrow and trabeculae will be replaced with more mature lamellar bon
Figure 1.2 Intramembranous ossification. (A) Small groups of mesenchymal cells cluster together to form an ossification center. (B) The mesenchymal stem cells differentiate into osteoblasts and synthesize osteoid tissue to form the early bone matrix. Osteoblasts become embedded in their own matrix and further differentiate into osteocytes. (C) Mineralization occurs forming trabecular bone. Blood vessels infiltrate the woven bone and mesenchyme cells condense to form the bone lining periosteum. (D) Intramembranous ossification concludes with the formation of mature bone, consisting of an outer compact layer and inner spongy bone filled with red marrow. Modified from © 2004 Pearson Education, Inc., published by Benjamin Cummings.
1.4.2 Endochondral ossification

Endochondral ossification is the process by which bone tissue is formed from pre-existing cartilage. This process begins in the second month of development mesenchymal stem cells cluster to form prechondrogenic nodules (figure 1.3 A). This process is driven by a variety of regulatory signals, ECM-cell interactions, and cell-cell interactions. Sox9 (sex determining region Y-box 9) is considered a master regulator of this process and expressed in precartilaginous tissue. (Bi et al., 1999). Mutations to the sox9 gene result in the rare skeletal disorder camptomelic dysplasia (Wright et al., 1995). Mesenchymal cell-cell interactions are driven by N-cadherin expression, as shown in embryonic limb development (Oberlender and Tuan, 1994). Upon differentiation, these now chondrocyte cell masses will begin to produce the matrix that forms the hyaline cartilage model. The cartilage model expands in length as cells continue to divide and secrete matrix components. The matrix consists of collagen types II, IX and XI, and proteoglycan aggrecan and perlecan. Collagen type II is the main component of cartilage and is required for growth plate formation (Li et al., 1995). Sox9 regulates the expression of type II collagen gene (col2a1), a key maker of chondrocytes (Bell et al., 1997; Lefebvre et al., 1997; Leung et al., 1998; Ng et al., 1997). Surrounding the hyaline cartilage is the perichondrium, which is composed of an outer fibrous layer containing fibroblast and an inner chondrogenic layer that contains progenitor cells. Homeobox (hox) genes regulate the formation of the perichondrium, through the regulation of runt-related transcription factor 2 (runx2) (Villavicencio-Lorini et al., 2010). The perichondrium will eventually develop into the periosteum, where the inner layer becomes the source of osteoblasts. These osteoblasts produce osteoid, a nonmineralized organic bone matrix that forms the bony collar surrounding the
cartilage model (figure 1.3 B). High levels of perlecan, along with heparanase, are detected along the edge of developing bone collar (Brown et al., 2008). Heparanase activity releases perlecan bound heparin-binding growth factors to drive osteogenesis. (Brown et al., 2008; Ishijima et al., 2012). As the periosteum develops, chondrocytes at the center of the cartilage template will approach the end of their life cycle and begin to calcify their surrounding matrix. This site is known as the primary ossification center. As cartilage begins to degrade, hollow cavities are formed and vasculature invades. Blood vessels, originating from the periosteum, invade through the developing compact bone matrix and create channels, called foramen, that facilitate the invasion of bone cells, nerves and nutrients to the inner hollow medullary cavity (figure 1.3 C). Remaining cartilage is eventually broken down and replaced by bone. Secondary ossification centers develop at the epiphysis, the rounded end at the joint of long bones, with the invasion of blood vessels. Bone growth occurs at epiphyseal plate, or growth plate, which is located where the diaphysis meets the epiphysis (figure 1.3 D). The chondro-osseous junction is located at the base of the epiphyseal plate where advancing bone tissue meets the chondral surface.
Figure 1.3 Endochondral bone formation. The majority of bone is created from a hyaline cartilage model. (1) The process begins with the condensation of mesenchymal stem cells to form an ossification center. (2) As these cells differentiate into chondrocytes they begin to degrade their surrounding matrix to form hollow cavities. At the same time the periosteal bone collars begins to enclose the cartilage bone tissue. (3) Blood vessels begin to invade the medullary cavity and the epiphyseal ends, where secondary ossification centers develop. (4) Bone continues to grow longitudinally at the epiphyseal plate. In the end, the cartilage model is replace by bone tissue. The outer surface of bone is composed of hard compact tissue while the inner tissue is more spongy and porous. Illustrations were adapted from Servier Medical Art by Servier.
1.4.3 Growth plate organization

The epiphyseal plates is composed of four different layers known as the resting, proliferating, prehypertrophic and hypertrophic zones (figure 1.4 A). Each zone contains chondrocytes at various stages of maturation. The resting zone, the outermost layer from the chondro-osseous junction, contains the source of chondrocyte progenitor-like cells for subsequent proliferative and differentiation. These cells have a distinct round morphology. Indian hedgehog (Ihh) induces differentiation of the chondrocytes in the resting zone (Kobayashi et al., 2005; Koziel et al., 2005). In the proliferation zone, the progenitor cells proliferate and arrange into columns that run with the direction of bone growth. The chondrocyte will change in appearance from a round shape to a more flattened cell body. At the prehypertrophic zone, mature chondrocytes exit the cell cycle and begin hypertrophic differentiation. At the hypertrophic zone, chondrocytes grow in size and undergo a terminal differentiation process that leads to cartilage calcification. Hypertrophic differentiation is characterized by *ihh* and *coll10a1* (Long et al., 2001; Zheng et al., 2003). Perlecan is found at along critical bone-cartilage borders, such as the chondro-osseous interface, perichondrium, and periosteum (figure 1.4 B). At these sites, perlecan acts as a barrier preventing encroachment of the bone tissue (Brown et al., 2008). Loss of perlecan disrupts the growth plate layers and leads to failure of the chondro-osseous junction (figure 1.6) (Ishijima et al., 2012).
Figure 1.4. Growth of long bone at the epiphyseal plate. The epiphyseal growth plate extends the diaphysis of bone longitudinally. (A) The growth plate consists of four principal layers of chondrocytes organized by stage of differentiation. Beginning at the top, furthest away from the diaphysis, is the resting zone (Rz), followed by proliferative zone (Pz), prehypertrophic zone (PHz), and the hypertrophic zone (Hz). The chondro-osseous junction is the interface between the hypertrophic zone and mineralized bone tissue. (B) Expression of perlecan and heparanase at the epiphyseal plate. Perlecan (red) and heparanase (green) were found at the chondro- of the developing mouse femur (E18.5) by immunostaining. Yellow arrow indicates COJ boundary and interface between intact perlecan and heparanase digested. Perlecan is present along the perichondrium (Pc) and periostium (Po). Nuclei are stained in blue. Figure B is a modified illustration from Servier Medical Art by Servier and from Ornitz & Marie (2002). Figure B was modified from Brown et al. (2008).
1.5.0 Bone remodeling and mechanics of mineralization

Bone is a highly active tissue that continually undergoes remodeling to maintain its structural integrity and regulate mineral homeostasis. Bone remodeling is a process where old mature bone is broken down (bone resorption) and replaced with new bone tissue (bone formation). Interestingly, the adult human skeleton is replaced fully about every ten years (Watts, 1999). Remodeling occurs at random sites of the skeleton, but is also a process by which bone fractures are healed (Burr, 2002; Parfitt, 2002). The following section will describe the principal cells of bone tissue and outline the five phases of bone remodeling: activation, resorption, reversal, formation and termination.

1.5.1 Bone cells involved in bone remodeling

Bone homeostasis requires dynamic coordination of three major osteogenic cell types acting in concert with the osteolytic cell type, the osteoclast: osteoblasts, bone lining cells and osteocytes (figure 1.5). Osteoclasts are responsible for bone resorption, initiating the process of bone remodeling. Although found at the bone surface these cells are of hematopoietic origin (Ash et al., 1980). A mature osteoclast cell is formed by the fusion of several monocyte-derived cells, which creates a single multinucleated cell that is relatively large in size compared to other bone cells (Vignery, 2005). A distinguishing feature of osteoclasts is their expression of tartrate-resistant acid phosphatase (Kirstein et al., 2006). The process of bone removal requires the secretion of acids and collagenases by osteoclasts. Osteoclasts are considered a terminally differentiated cell (Arai et al., 1999). Rather than undergo mitosis, osteoclasts are replenished by cells in circulation (endosteal sinus). On the other side of the spectrum are the osteoblasts, mesenchymal stem cell-derived cells, that are responsible for the formation of bone (Aubin, 1998). A primary
source for osteoblasts is the perichondrium layer on the outside surface of bone (Kronenberg, 2007). Unlike osteoclasts, osteoblasts are capable of mitotic division (Buckley et al., 1988; S. Li et al., 2007). Compared to osteoclasts, osteoblasts are much smaller and only contain one nucleus. Osteoblasts produce the major components of the organic bone matrix, including cross-linked type 1 collagen, osteocalcin, osteonectin and osteopontin. Mineralization occurs as the osteoid integrates with the inorganic matrix and precipitates. Once the formation of bone is complete the osteoblasts will either differentiate into a bone lining cell or osteocyte or undergo apoptosis (Dallas and Bonewald, 2010). Bone lining cells, as name suggest line the exterior surface of bone. These cells differ from osteoblasts in terms of morphology, function, and expression of proteins (Everts et al., 2002). Although not thought of as a major component of the remodeling process, evidence suggest the bone lining cells play an important role in coupling bone resorption to bone formation (Everts et al., 2002). Osteocytes make up approximately 90% of the total cells in bone tissue (Schaffler and Kennedy, 2012). Osteocytes are derived from osteoblasts that have become embedded in their surrounding bone matrix. Osteocytes project long dendrite-like processes that extend throughout the bone matrix and facilitate cell-cell communication (Bonewald, 2007). These cells are considered relatively inert with reduced synthetic activity. Osteocytes do not undergo mitotic division. A distinguishing characteristic of osteocytes is the ability to secrete sclerostin, a glycoprotein that inhibits bone formation (van Bezooijen et al., 2005). Osteocytes also produce dentin matrix protein 1 (DMP1), phosphate-regulating neutral endopeptidase on chromosome X (PHEX), matrix extracellular phosphoglycoprotein (MEPE), fibroblast growth factor 23 (FGF-23), osteoprotegerin (OPG) and receptor
activator of nuclear factor-κB (RANKL) (Schaffler and Kennedy, 2012). Osteocytes play an important role in the turnover of the bone matrix through mechanosensory mechanisms (Skerry et al., 1989). The role osteocytes play in mechanosensing will be discussed in section 1.6.
Figure 1.5 Bone remodeling process. Schematic diagram representing the stages of bone resorption and bone formation. In sequential order, the stages of bone remodeling are: 1) activation, 2) resorption, 3) reversal, 4) formation, and 5) termination. Bone resorption is initiated by the recruitment of pre-osteoclasts, which will attach to bone surface and begin to break down old bone. The reversal cell, a cell of unknown origin, couples bone resorption to bone formation. Osteoblasts, derived from mesenchymal stem cells, are responsible for creating new bone tissue. Mature osteoblasts can further differentiate into either bone lining cells or osteocytes. Bone lining cells are quiescent and remain on the outer surface of bone. Osteocytes are formed when osteoblasts become embedded in their surrounding matrix. Illustrations were adapted from Servier Medical Art by Servier.
1.5.2 Osteoclast activation

Activation phase begins with the recruitment of osteoclast precursors, or mononuclear monocyte-macrophages cells, from circulation to site of remodeling. The earliest known hematopoietic precursors able to form osteoclasts are the colony forming unit granulocyte-macrophage (CFU-GM), thought to be a monocyte-like cell, while other CFUs form osteoclasts but at a much lower efficiency (Boyle et al., 2003). The definitive physiological in vivo osteoclast precursor cell type is still not known. Macrophage-osteoclast differentiation is regulated by a number of transcription factors, including PU.1, Mitf (microphthalmia-associated transcription factor), and c-FOS. PU.1 is highly expressed in macrophages and neutrophils, and is an essential signaling molecule for osteoclastogenesis (Anderson et al., 1999; Dahl et al., 2003). In fact, mice null for PU.1 lack the ability to produce osteoclasts at all (Tondravi et al., 1997). PU.1 also regulates the transcription of the gene RANK, for receptor activator of nuclear factor κ B, whose activity is important for osteoclast differentiations, resorption activity, and cell survival (Kwon et al., 2005). RANK osteoclastic activity is triggered by RANK-Ligand (RANKL), which is bound to the osteoblasts surface. MITF, a member of the helix-loop-helix-leucine zipper protein family, is expressed in macrophages, osteoclasts and associated precursor cells and is important for the survival and differentiation of osteoclasts (Luchin et al., 2000). The interaction between PU.1 and MITF regulates a number of genes during osteoclast differentiation, including cathepsin K (ctsk), acid phosphatase 5 (ACP5) and osteoclast-associated receptor (OSCAR) (Hu et al., 2007; Luchin et al., 2001; So et al., 2003). c-FOS is a leucine zipper protein that dimerizes with proteins of the jun family to form the transcription factor complex AP-1 (Chiu et al.,
As such, this transcription complex can influence cell proliferation, differentiation, and transformation. Mice lacking c-fos develop bone disease osteoporosis (Grigoriadis et al., 1994). A c-fos-deficiency inhibits macrophage-osteocyte differentiation, and relative to PU.1-deficient mice inhibition occurs at a later stage (Grigoriadis et al., 1994). PU.1 regulates the transcription of the macrophage colony-stimulating factor receptor (M-CSF) (Zhang et al., 1994). Activation of M-CSF promotes osteoclast cell survival, drives cellular proliferation, and induces cytoskeletal reorganization.

Remodeling occurs at sites of bone tissue damage, caused by mechanical strain, or can be triggered by the release of hormones, such as calcitonin, parathyroid hormone (PTH), or estrogen. Secretion of calcitonin and Pth, along with vitamin D₃, control serum calcium levels (Crockett et al., 2011). Calcitonin inhibits osteoclastic resorption. PTH is a calcitropic hormone secreted by the parathyroid glands when serum calcium is low. Released PTH then will activate the PTH receptor, a seven-transmembrane G-protein-coupled receptor, expressed on the surface osteoblastic cells (Juppner et al., 1991). This in turn activates protein kinase A, protein kinase C, and calcium signaling that ultimately produces a number of signaling molecules that recruit osteoclast precursor cells and drive osteoclast differentiation (Swarthout et al., 2002). PTH stimulates osteoblast expression of the chemokine monocyte chemoattractant protein - 1 (MCP-1), which stimulates preosteoclasts recruitment and drives promotes monocyte fusion (X. Li et al., 2007). PTH also modulates the osteoblast expression of CSF-1, RANKL, and OPG, which target receptors on the surface of osteocytes (Insogna et al., 1997; Ma et al., 2001). Osteocytes normally secrete basal levels of transforming growth factor beta (TGF-β) that inhibits
osteonogenesis (Heino et al., 2002). However, changes in mechanical load can influence TGF-β secretion (Bonewald, 2007). Bone damage or weightlessness can lead to osteocyte apoptosis and therefore lower TGF-β levels, which promotes osteoclastogenesis (Aguirre et al., 2006; Verborgt et al., 2002). The sex hormone estrogen triggers preosteoclast and osteoclast apoptosis, hence, decreased levels of estrogen in women after menopause results in osteoclasts formation and survival, leading to higher rates of bone loss (Nakamura et al., 2007; Väänänen and Härkönen, 1996; Zaman et al., 2006).

1.5.3 Bone resorption

At the resorption phase, the monocyte-like precursors fuse together and form multinucleated preosteoclasts (Vignery, 2005). These large multinucleated preosteoclasts will attach to the bone matrix through interactions between integrin and RGD (arginine, glycine, and asparagine) containing matrix proteins, such as osteopontin and bone sialoprotein (Ross et al., 1993; McHugh et al., 2000;). Osteoblasts facilitate osteoclasts attachment by secreting matrix metalloproteinases (MMPS), such as MMP-2,-3,-9 and -13, to degrade bone matrix lining and expose RGD adhesion sites (Kusano et al., 1998). The area underneath the attached osteoclasts is referred to as the sealed zone. Mature osteocytes pump out hydrogen ions, via H-ATPase proton pumps and chloride channels, to lower the pH of the sealed zone (Silver et al., 1988). Bone tissue is broken down by a variety of factors secreted by osteocytes including tartrate-resistant acid phosphatase, cathepsin K, and MMPS (Atley et al., 2000; Bianco et al., 1988; Bord et al., 1998; Fuller and Chambers, 1995; Zeitler et al., 2004). This begins the dissolution of the bone matrix and produces a groove or cavity at the bone surface, known as Howship’s resorption lacunae. At the end of the resorption phase, osteoclasts undergo apoptosis and
the Howship lacunae is covered with a thin layer of collagenous matrix (Everts et al., 2002).

1.5.4 Reversal phase

The reversal phase begins with removal of the collagenous matrix from the Howship lacunae. This clearing is achieved by “reversal” cells (Delaisse, 2014). The origin of these cells is still unclear. One thought is that they are of monocyte descent, which was concluded based on morphology (Tran Van et al., 1982). The other proposed idea is that they are of the osteoblast lineage (Andersen et al., 2013). To further complicate the matter, both monocyte-derived cells and osteoblasts can express some of the same markers (Heinemann et al., 2000; Kraal et al., 1987). Additionally, both macrophages and osteoblasts produce MMPs, necessary for breaking down bone tissue, and osteopontin, a major component of the mineralized matrix (Newby, 2008; Takahashi et al., 2004). On the other hand, an osteoblasts lineage seems to be ideal considering they produce the collagen matrix (Everts et al., 2002). Further linear tracing must be conducted to assess the true origin of these cells. In any case, it is thought the reversal cells couple bone resorption to bone formation.

1.5.5 Bone formation

During the formation phase, preosteoblasts are recruited to the resorption lacunae where they further differentiate and secrete bone matrix, primarily collagen type I. The other organic components are various proteoglycans and glycoproteins, small integrin-binding ligands proteins, gamma-carboxyglutamic acid-rich (Gla) containing proteins like osteocalcin, and lipids (Clarke, 2008). Hydroxyapatite is the main inorganic material found in bone. Complete bone mineralization requires nonspecific alkaline phosphatase,
nucleotide pyrophosphatase phosphodiesterase, and progressive ankyloses protein. A number of signaling molecules are thought to link bone resorption to bone formation, although the exact mechanism remains unknown. TGF-β1 is an attractive coupling candidate, as it released during bone resorption signals for the migration of preosteoblast mesenchymal stem cells to resorption lacunae (Tang et al., 2009). Other reported candidates include insulin-like growth factors (IGFs), bone morphogenetic proteins (BMPs), platelet-derived growth factor, and FGFs (Bonewald and Mundy, 1990; Hayden et al., 1995; Hock et al., 1988; Ikeda and Takeshita, 2014; Locklin et al., 1999). It has been proposed that osteoclasts produce the coupling factor(s) that prompts bone formation. One study found that the induction of osteoclast derived chemokine sphingosine 1-phosphate and BMP6 stimulates human mesenchymal stem cell migration and differentiation towards osteoblast lineage (Pederson et al., 2008). Osteoblast differentiation is also achieved through the osteoclasts ligand ephrin-B2 which signals through the osteoblast ephrin type-b receptor 4 (Zhao et al., 2006).

1.5.6 Termination phase

Bone remodeling process concludes when the resorbed bone has been completely replaced and the resting bone surface has been reestablished. At this point, osteoblasts have undergo one of the following fates: apoptosis, revert to a quiescent bone-lining cell, or remain embedded in the matrix and become an osteocyte. Sclerostin, a bone morphogenetic protein antagonist, serves as an attractive termination signal for the remodeling cycle (Sapir-Koren and Livshits, 2014). If this were the case one would expect sclerostin levels to inversely correlate with bone formation. Interestingly, sclerostin expression decreases at the transition period coupling bone resorption to bone
formation. Some researchers argue there is no active terminator, instead, remodeling ceases simply because signals are no longer being produced.

1.6 Bone adaptation to mechanical stimulation

Bone is a highly adaptive tissue that can rearrange its tissue architecture in response to the mechanical environment. It is well known that mechanical loading, such as weight-bearing exercise, can increase bone density and strength. On the other hand, prolonged non-weight bearing conditions, such as weightlessness in astronauts, casting for fractures, and bed rest have the opposite effect. To better understand this phenomenon, much research has been conducted using animal models under conditions of microgravity or various load/unload rates.

Currently, osteocytes are considered the major players involved in bone mechanosensing. Targeted deletion of osteocytes in mice disrupts the bone mechanosensing process and results in bone loss (Tatsumi et al., 2007). Osteocytes are distributed throughout the matrix of mineralized bone tissue. They project long cytoplasmic extension to form an interconnected network with one another and with cells at the bone surface (figure 1.6 A). This network is referred to as the lacuna-canalicular system. Osteocytes reside in non-mineralized fluid-filled spaces called lacunae (figure 1.6 B). Their cell processes create narrow channels, or canaliculi, which permit the flow of interstitial fluid throughout the bone matrix (figure 1.8 C). Weinbaum et al. (1994) proposed a model in which osteocytes were stimulated by fluid-flow shear stresses acting on their membrane surface (Weinbaum et al., 1994). Some of the earlier work leading to this model showed cyclic loading in bones produced fluid through the canaliculi and fluid-saturated bone created stress-generated potentials (PIEKARSKI and MUNRO, 1977; Pienkowski and Pollack,
1983). It was later found that oscillatory fluid flow resulted in bone formation (Qin et al., 2003). Fluid flow has been shown to stimulate osteocyte cells, resulting in increased intracellular calcium concentration, and the release of signaling molecules such as adenosine triphosphate, nitric oxide, sclerostin, prostaglandin, and osteoprotegerin, RANKL (Genetos et al., 2007; Jiang and Cherian; Klein-Nulend et al., 1995; Spatz et al., 2015; You et al., 2008). The mechanism by which osteocytes sense mechanical strain and translate these forces into a cellular response is not entirely understood (Temiyasathit and Jacobs, 2010). As first proposed by (You, Weinbaum, Cowin, & Schaffler, 2004), it appears that the pericellular matrix (PCM), a thin proteoglycan-rich glycoalyx lining the cell surface, is facilitate osteocyte regulates mechanosensing (figure 1.8 C) (You et al., 2004) identified transverse tethering elements that anchored the cell process to the bone tissue wall. It is thought these tethers serve as antennas to the osteocyte cell and sense the mechanical environment. Perlecan was identified as a tether element in the LCS and shown to be important for maintaining the canaliculi structural integrity by preventing encroachment of the mineralized matrix wall (Thompson et al., 2011). Furthermore, in perlecan deficient mice, the reduced levels of perlecan within the LCS interfered solute transport and mechanosensing ((Wang et al., 2014). Decreased perlecan expression in bone lead decreased bone quality and phenotype resembling osteoporosis (Lowe et al., 2014).
Figure 1.6 Lacunar-canalicular system of cortical bone. (A) Osteocytes project long slender cytoplasmic processes that create narrow passage ways (canaliculi) for cells to interact and communicate. (B) The lacuna is mineralized space where individual osteocytes reside. The space between the cell body and bone allows for interstitial fluids and nutrients to pass through. (C) The canaliculus is formed around the projecting cytoplasmic cell process. Between the cell process and the mineralized bone wall is the pericellular space is the area, where fluid flows. Lining the osteocyte cell process is the PCM, which is rich in proteoglycans. Linear tethers transverses the pericellular space and maintains the canalicular structure.
1.7 Common clinical diseases and disorders affecting the skeletal tissues

The human skeletal system is susceptible to disease. Damage to bone and associated tissue affect motion, cause severe pain, decreases the quality of life, and can be life threatening. Osteoporosis and arthritis are two of the most common diseases affecting the skeletal system with great economic impact. According to the National Institute of Arthritis and Musculoskeletal and Skin Disease it is estimated that over 53 million Americans already have osteoporosis or are at high risk and approximately 50 million Americans suffer from some form of arthritis. Osteoporosis is a disease that results in decreased bone mineral density (BMD), reduced bone quality, and increased fracture occurrence (Seurer and Huntington, 2015). There are ways to improve bone strength and health in individuals with osteoporosis, although treatments options can be very expensive. The most common types of arthritis are osteoarthritis and rheumatoid arthritis (Clements, 2011; Sarzi-Puttini et al., 2005). Osteoarthritis is the degeneration of the cartilage at the joints. This leads to the formation of bone spurs/osteophytes, joint narrowing, stiffness, swelling, and chronic pain. Rheumatoid arthritis is an autoimmune inflammatory condition when the body attacks its own joints, causing destruction, painful swelling, and bone erosion. Treatment options for arthritis are very limited and only offer temporary relief.

The skeletal system is affected by a number of genetic disorders, although they are individually rare. Together, these disorders constitute a large group of clinically distinct and heterogeneous conditions. Clinically, skeletal disorders have been categorized as either dysostoses or osteochondrodysplasias. Dysostoses, is defined as a developmental abnormality affecting the bone itself, whereas osteochondrodysplasias, describes a disorder affecting both bone and cartilage tissue, as in the joints. Patient symptoms often vary,
making these disorders difficult to diagnosis and understand their pathogenesis. Even with
the same molecule origin clinical outcomes differ significantly. Mutations to collagen I
and II, major components of bone and cartilage respectively, produce various skeletal
defects. Osteogenesis imperfecta (OI), is a rare disorder, for which there is no cure, caused
by mutations to the col1A1 and col1A2 genes (OMIM #166200) (Prockop, 1990).
Individuals with severe OI have brittle bones that are prone to fracture. Mutations in
col2A1 can lead to various bone dysplasias, including achondrogenesis type II (OMIM
#200610), an embryonic lethal condition characterized by major bone defects, and Stickler
dysplasia (OMIM #108300), which results in a more mild growth impairment and distinct
facial features (Steiner et al., 2013). Not surprisingly, mutations to genes coding for
connective tissue ECM proteins tend to produce similar phenotypes. Both col10A1 and
colL11A2 are linked to skeletal dysplasias (Ikegawa et al., 1998; Melkonieni et al., 2000).
Aggrecan is associated with two disorders, spondyloepiphyseal dysplasia-Kimberly type
(OMIM #608361) and spondyloepimetaaphyseal dysplasia, aggrecan type (OMIM
#612813) (Gleghorn et al., 2005; Tompson et al., 2009). Heterozygosity for mutations in
cartilage oligomeric matrix protein (COMP, OMIM #177170) result in
pseudoachondroplasia and multiple epiphyseal dysplasia (Briggs et al., 1995). In fact, most
reported cases of pseudoachondroplasia have a COMP mutation (Park et al.). On the other
hand, multiple epiphyseal dysplasia can also be associated with mutations to col9A1,
col9A2, col9A3, and matn3 (matrilin 3) (Park et al.). Similar abnormalities are seen in
patients with mutations to the perlecan gene, HSPG2, which result in two classes of skeletal
disorders: Schwartz-Jampel syndrome (SJS, OMIM #255800) and dyssemental
dysplasias, Silverman-Handmaker (DDSH, OMIM #224410) (Arikawa-Hirasawa et al.,
2002, 2001). SJS and DDSH will be discussed in more detail in section 1.9.5. Krakow & Rimoin (2010) have a comprehensive review article about skeletal dysplasias and provide a thorough list of well characterized disorders with identified molecular etiologies. The point of this passage is to draw attention to the importance of the ECM to bone health. Understanding the role of each of these ECM components will aid in the development of effective therapeutics.

1.8.0 An overview of cartilage biology

In many ways bone is impacted by cartilage. As mentioned in the earlier passage, most of the human skeleton is created from cartilage template and in mature tissues cartilage covers the ends of all bones that meet at a joint. To no surprise, conditions that interfere with normal cartilage development or function ultimately impact bone. Considering the interconnectedness of these two tissues it is important to understand the relationship between cartilage and bone. In this section the physiological function of cartilage will be described and current cartilage engineering strategies will be discussed.

1.8.1 Cartilage function in adult tissues

In adults, cartilage functions as a flexible yet strong weight-bearing support for bone joints, the rib cage, the vertebral discs and many other tissues. Cartilage made up of specialized cells called chondrocytes, which produce a large amount of ECM that is rich in collagen fibers and proteoglycans. Unlike most connective tissues, mature cartilage is avascular and thus possesses poor regeneration capacity. Factors such as age, injury, and disease contribute the effectiveness of normal cartilage function (Akizuki et al., 1987). There are three types of cartilage: hyaline, elastic and fibrocartilage (Frisenda et al., 2013). Apart from their function and location within the human body, these cartilages
differ in terms of tissue organization and ECM composition. Hyaline, the most abundant of the three, covers the articular surfaces of bone at synovial joints, connects the ribs to the sternum, and supports the nose, ears, and larynx. Hyaline cartilage is a strong, flexible tissue with a glossy appearance. The primary function of hyaline cartilage is to bear and distribute mechanical loads (Bhosale and Richardson, 2008). Elastic cartilage is more flexible than hyaline, and found in the external portion of the ear and covering the opening of the larynx. Fibrocartilage is a tough, inflexible tissue with high tensile strength (Cox and Peacock, 1977). Fibrocartilage functions to alleviate pressure as seen in the knee and intervertebral disc (Benjamin and Evans, 1990).

1.9.2 Current trends in cartilage tissue engineering

Most people will experience cartilage damage at some point in their life, if not from disease, then from injury or normal wear and tear associated with aging. For these reasons, it is important to develop strategies to heal or replace damaged tissue. Doing so would have a tremendous positive economic impact. Current therapies only provide temporarily relief, however, stem cell-based approaches appear promising and are currently favorable in the field tissue engineering (Cucchiarini et al., 2015). Autografting method involves isolating stem cells directly from the patient, growing these cells in an in vitro system, and then reintroduce back into the patient at the site of disease or injury to repair/replace the damaged tissue. A successful outcome would be that these cells integrate into the native tissue, produce the desired tissue matrix components, and provide functional mechanical properties (Green et al., 2015; Zhang et al., 2016).

Mesenchymal stem cells (MSCs), a heterogeneous subset of stromal cells, are the most attractive source for clinical application (Reissis et al., 2016; Scarfì, 2016; Yin et al.,
MSCs can be isolated from various connective tissues including, bone marrow, skeletal muscle, adipose tissue, synovium, etc. Once isolated, these cells can be expanded in vitro and differentiated into almost any mesodermal lineage. The source of MSCs used in clinical studies so far include bone marrow-derived MSCs, adipose-derived MSCs, and umbilical cord blood MSCs, and peripheral blood MSCs (Howard et al., 2008). It remains to be discovered which cell source has the best chondrogenic potential. The chondrogenic potential of MSCs has been tested extensively in in vitro settings (Wang et al., 2015; Yin et al., 2016). However, there is not a consensus method, i.e. cell medium condition and growth factor supplementation, to drive chondrogenic differentiation. Many methods exist to induce chondrogenesis. Typically, the output for these studies is the production of proteoglycans and type II collagen. Some of the common supplements used to induce chondrogenesis include TGF-β1 and TGF-β3, IGF-1, and BMP-2 (Brady et al., 2015). Together, all these factors create a very complicated system. This is why researchers must continue to investigate the basic biology of these cells. The use of cell lines have been instrumental in discovering key aspects of the chondrogenesis process. The most used chondrogenic cell models are the ATDC5 cells, a chondrogenic cell line derived from a mouse teratocarcinoma, and CH310T1/2 cells, derived from a C3H mouse embryo (Atsumi et al., 1990; Reznikoff et al., 1973). A major obstacle in cartilage engineering is obtaining functional integration of the engineered tissue. The ideal system is one that conveys the right biochemical and mechanical signals. For the system itself, we know the composition, structure and mechanical properties can drive chondrocyte fate, regardless of the stimulating factors present (Panadero et al., 2016). Currently, most researchers favor a 3D system made of a
biocompatible material with native tissue components incorporated. Current 3D scaffolds are made from components such as silk fibroin/chitosan, collagen hyaluronic acid, agarose, and alginate, and (Dinescu et al., 2015; Ghezzi et al., 2015; Snyder et al., 2014; Sridharan et al., 2015; Vishwanath et al., 2016). Techniques that model mesenchymal condensation, as occurs in early cartilage formation, are becoming more popular and have shown promising results. For example, used a poly(l-lactide-co-glycolide)/poly(l-lactide) microfiber scaffolds coated with human fibroblast-derived matrix to culture MSCs (Kim et al., 2016). Using this scaffold resulted in cell-cell adhesion through focal adhesion kinase (FAK) involvement and upregulated chondrogenic markers N-cadherin and neural cell adhesion molecule. Others have used centrifugation to form condensed mesenchymal bodies (Bhumiratana et al., 2014). In the end, the most successful approaches will be the ones that take cues from nature.

Perlecan, a major component of cartilage, is often overlooked in many of today’s current models, despite its recognized importance for cartilage formation (Arikawa-Hirasawa et al., 1999; Farach-Carson et al., 2014a). Section 1.9 will provide a review of perlecan biology and its specific role in chondrogenesis will be examined in chapter 4.

1.9.0 Perlecan/HSPG2

Perlecan is essential for skeletal development, maintenance and function (Hassell et al.). Perlecan acts as a barrier, physically separating cells from their bulk matrix environment (Farach-Carson et al., 2014a). This dissertation explores perlecan’s role along cell interfaces, specifically within bone and cartilage. The following sections will provide general information about perlecan and define its role in preserving skeletal tissue integrity.
1.9.1 Protein domain structure

The human gene *HSPG2*, located on chromosome 1p36, codes for the secreted basement membrane (BM) proteoglycan, perlecan. Perlecan is also a key component of musculoskeletal tissues such as bone, cartilage and muscle (Farach-Carson and Carson, 2007). Human perlecan consists of 4,391 amino acids and has molecular weight of 470 kDa, with additional glycosaminoglycan (GAG) side chains capable of extending the molecule to over 700 kDa (Graham et al., 1999; Kimura et al., 1999; Murdoch et al., 1992; Whitelock et al., 1999). This makes perlecan one of the largest matrix molecules. The protein core contains five modular domains, referred to as domains I-V (figure 1.7) that are arranged in a linear fashion resembling beads-on-a-string (Paulsson et al., 1987). Perlecan’s domains share homology to a number of different ECM proteins. Surprisingly, perlecan is almost always processed into its full-length protein form, with only a few human variants being reported (i.e. *miniperl GenBank AAL79552.1*: Jung et al., 2013).
Figure 1.7 Domain structure of Perlecans. Perlecans consists of five distinct domains (I-V). The protein core itself is a linear arrangement of repeating modular units that share homology to a number of different protein families. The core protein contains three GAG attachment sites in domain I and a single variably glycosylated site at its c-terminal end domain V. Modified from Farach-Carson and Carson (2007).
Domain I contains a Sperm, Enterokinase and Agrin (SEA) fold, which is found in other matrix and cell surface proteins (Bork and Patthy, 1995; Dolan et al., 1997). To date no specific function of this fold has been demonstrated but it is known to be associated with protein regions that are highly glycosylated. In fact, domain I contains three GAG attachment sites that precede the sperm protein, enterokinase and agrin (SEA) fold (Dolan et al., 1997). Perlecan GAGs can be composed of either heparan sulfate or chondroitin sulfate depending on the cell source (Dolan et al., 1997; Graham et al., 1999). This seems to be depend on the cell source and the tissue in which the protein is being expressed in. Heparin binding growth factors involved in key development processes are associated with the GAG side chains of perlecan. During wound healing, perlecan’s GAG chains are cleaved, releasing growth factors and cytokines directly at the site of injury (Zhou et al., 2004). Domain II contains four low density lipoprotein (LDL) receptor motifs and one isolated immunoglobulin-like (IG) fold (Murdoch et al., 1992). Both of these structures are held together with disulfide bonds and contribute to the compact modular shape of perlecan (Costell et al., 1996). It is likely this domain is involved in LDL and calcium binding. It is also speculated that domain II is involved in wingless (Wnt) signaling, which is important for many developmental processes (Farach-Carson and Carson, 2007). Perlecan’s modulation of Wnt has been demonstrated in various vertebrates (Kamimura et al., 2013). Domain III two contains both laminin epidermal growth factor (EGF) laminin IV type A (laminin B) domains (Schulze et al., 1995). Domain III forms an inflexible rod-like structure that is maintained by disulfide linkages in the laminin EGF domains. Fgf-18, found in developing growth plates and
possesses mitogenic activity on chondrocytes, binds directly to domain III of perlecan (Smith et al., 2007). The largest and, until recently, least studied region of perlecan is domain IV, spanning about half the molecule (Whitelock et al., 1999). In humans domain IV is made up of 21 repeating Ig (C2-type) modules that are tandemly linked together. In general, Ig motifs are important for protein-protein interactions, and found in many ECM and cell surface proteins. Domain IV of perlecan is known to interact with many ECM components including nidogen and fibronectin (Hopf et al., 1999). Proteins containing Ig repeats have been linked to providing mechanical stability to flexible tissues such as cartilage and muscle (Improta et al., 1996; Marín et al., 2003). Typically these proteins are large in size and modular. Only recently has perlecan been examined in this context. Interestingly, mice only have 14 Ig modules while most other mammals have 21 (Noonan et al., 1991). It is likely some gene insertion or duplication event occurred in humans (Farach-Carson et al., 2014a; Warren et al., 2015). Domain V contains three laminin G, two EGF domains and has the fourth GAG attachment site (Murdoch et al., 1992). Domain V can be enzymatically processed to produce a fragment, known as endorepellin (Mongiat et al., 2003). When left intact perlecan possesses proangiogenic properties, while the fragment endorepellin has antiangiogenic activity (Zoeller et al., 2009). Endorepellin has largely been studied for its role in controlling tumor angiogenesis and in neuroprotection following ischemic stroke (Douglass et al., 2015; Marcelo and Bix, 2014).

1.9.2 Conservation of HSPG2

Perlecan is a highly conserved molecule that has been studied in many model organism including mice, zebrafish, chicken, *C. elegans, Drosophila* and *N. nematostella.*
Interestingly, the mouse version of perlecan only contains 14 Ig modules in its domain IV, whereas humans and many other mammals have 21 (Murdoch et al., 1992; Noonan et al., 1991). This likely resulted from an alternative splicing event (Farach-Carson et al., 2014a; Warren et al., 2015). The protein identity for this domain remains nearly 90 % amongst mammals (Farach-Carson et al., 2014a). Other species differ in terms of number of Ig modules and their composition, with Gallus containing 18, Danio rerio with 12, and C. elegans with 15. See figure 1.8 for a comparison of domain IV between common model organisms. The mouse form of perlecan also contains a unique integrin-mediated RGD binding site within its domain III that is not found in human perlecan (Chakravarti et al., 1995). Both Drosophila and C. elegans do not have GAG attachments sites in their domain I. Although, Drosophila do retain the SEA fold (Hummel et al., 2004). Recent effort has been put forth to study the evolutionary origin of the perlecan gene in hopes of understanding these differences among species and to gain insight into its role in establishing tissue layers (Warren et al., 2015). In this recent study, the perlecan gene was identified in the genome of early metazoans, considered to have relatively simple tissue structures. In silico work revealed the perlecan gene was conserved in the genomes of placozoan and cnidarian, and to a lesser extent in poriferan. The perlecan gene was not found in the genomes of ctenophore or capaspora owczarzaki. These findings were validated in cnidarian N. vectensis and the placozoan T. adhaerens using polymerase chain reaction (PCR) methods. N. vectensis and T. adhaerens are considered morphologically simple animals, and have been used to study tissue regeneration and evolution of multicellularity, respectively. The perlecan gene in T. adhaerens is encoded by two separate, but adjacent genes, that house, in order, all five domains of the human
gene. In *N. vectensis*, the perlecan gene was detected in cells forming key tissue boundaries, and was activated during wound healing and the formation of new BMs. Given perlecan’s complex gene structure, it is surprising this molecule has remained conserved over millions of years of evolution. This suggest some ancient key function, likely evolving with the emergence of tissue layers (Farach-Carson et al., 2014a; Warren et al., 2015).
Figure 1.8 Comparison of domain IV in various species. The perlecan domain IV protein sequence was analyzed in *Homo sapiens*, *Mus musculus*, *Gallus*, *Danio rerio*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. The alignment was performed using the software Geneious v5.4 (Kearse et al., 2012). The top graph represents consensus sequence identity among the species examined. All sequences are compared to perlecan of *Homo sapiens*, which is highlighted in yellow at the top of the list. Sequences are shown in a grey color scale, representing low (white) to high (black) similarity to that of the reference protein. The purple arrows parallel the sequence of individual Ig modules.
1.9.3 Perlecan homologues

In *C. elegans*, the *hspg2* homologue is *unc-52* (Rogalski et al., 1993). Unc mutants were first discovered for their role within the body-wall of muscle cells (Brenner, 1974). *Unc-52* phenotype is characterized by retarded sarcomere construction and progressive paralysis, hence the name “unc” for uncoordinated movement (Mackenzie Jr. et al., 1978). Unlike human *hspg2*, *unc-52* codes for serval isoforms of perlecan. In general, longer isoforms are involved in the attachment of the myofilament lattice to the muscle cell membrane, while the role of shorter isoforms is not clear. *Unc-52* contributes to gonadogenesis through regulation of growth factor signaling and by providing structural support to the tissue surrounding the gonad (Merz et al., 2003). The *unc-52* depleted gonad BM interferes with gonadal cell adhesion signaling and affects the migration of gonadal leader cells (Gilchrist and Moerman, 1992; Merz et al., 2003). *Unc-52* mutations are associated with ECM remodeling defects in developing organs (Jafari et al., 2010).

Trol, for terribly reduced optic lobes, is the *Drosophila* homologue of the vertebrate protein perlecan, (Datta and Kankel, 1992). Trol regulates critical development signaling pathways, such as Wnt and IHH, and coordinates cell movement to establish cell and tissue layers. Trol is highly expressed in the developing central nervous system and the periphery, especially at the motor axon trajectories (Cho et al., 2012). Hence its name, Trol modulates semaphorin-mediated repulsive axon guidance in the optic lobes. It has been shown Trol can augment semaphorin suppression of focal adhesion kinase activation, suggesting Trol supports the antagonistic effect of semaphorin on integrin signaling (Cho et al., 2012). Similarly, during gastrulation Trol directs the movement of mesoderm cells to form a single mesoderm cell layer underlying the ectoderm.
This process requires cooperation of FGF signaling. Mutations to Trol not only modulate key signaling processes but also influence cell behavior through rearrange the territorial matrix. Such is the case seen during Drosophila hematopoiesis, where perlecan is expressed along the thin BM surrounding the blood progenitors. Here, perlecan regulates blood cell differentiation, through modulation of Hedgehog signaling, and structural supports the ECM of the lymph gland (Grigorian et al., 2013). Wnt signaling is also regulated by Trol and is important for formation of pre and postsynaptic structures of the neuromuscular junction (Kamimura et al., 2013).

1.9.4 Developmental expression of perlecan

Perlecan expression begins at the early stages of embryogenesis. Perlecan was detected along the cell surface of blastomeres at the two-cell stage and during the attachment phase of implantation at the exterior surface of the trophectoderm (Carson et al., 1993; Smith et al., 1997). Following implantation, perlecan accumulates throughout the developing cardiovascular system and at sites of cartilage primordia (French et al., 1999; Handler et al., 1997). At embryonic day 10.5 (E10.5), perlecan is found vascularized tissues such as the heart, pericardium, blood vessels walls, and in cartilage primordia (French et al., 1999; Handler et al., 1997). The highest deposition of perlecan occurs in cartilage undergoing endochondral ossification, such as the primordium of vertebral bodies and rib cartilage. At later stages of development, perlecan is expressed throughout the basal lamina of the embryo and organs such as the lung, kidney, liver, gastrointestinal tract and brain.

1.9.5 HSPG2 associated skeletal defects

A number of mutations have been identified in both Schwartz-Jampel syndrome (SJS; OMIM #255800) and dyssegmental dysplasia, Silverman-Handmaker type (DDSH; OMIM #224410),
but as of yet no exact genotype-phenotype correlations has been identified (Arikawa-Hirasawa et al., 2002, 2001; Rieubland et al.; Stum et al., 2006). However, the degree of severity inversely correlates with the amount of perlecan being deposited into the ECM (Stum et al., 2008). SJS is the relatively milder condition of the two characterized by myotonia and chondrodysplasia (Mereu et al., 1969; SCHWARTZ and JAMPEL, 1962). Mutations associated with SJS lead to reduced levels of normal perlecan secretion into the ECM (Arikawa-Hirasawa et al., 2002). SJS patients survive but experience widespread skeletal abnormalities including reduced stature, facial dimorphism, pigeon breast and shortened long bones. DDSH is neonatal lethal condition caused by functional null mutations to HSPG2 (Arikawa-Hirasawa et al., 2001; Rieubland et al.). Clinical features of DDSH include dwarfism, short and bowed limbs, flat facial features, anisospondyly, and encephalocele (Handmaker et al., 1977).

1.9.6 Mechanisms underlying perlecan developmental defects

The mechanism underlying perlecan disorders have been studied in mice. Perlecan-null mice, mimicking the DDSH phenotype, display normal formation of basement membranes during the first few days of development but these soon deteriorate at areas undergoing increased mechanical stress (e.g. contraction of the myocardium and expansion of brain vesicles (Arikawa-Hirasawa et al., 1999; Costell, 1999; Sasse et al., 2008). Perlecan-null mice typically die around embryonic day (E) 10-12, attributed to heart and brain defects. In the heart, the loss of perlecan weakens the basement membranes around the heart wall, leading to a “leaky” interface between cardiomyocytes and surrounding endothelial cells (Costell, 1999; Sasse et al., 2008). As a result, embryos die of heart arrest from blood leaking into the pericardial cavity. The cardiomyocytes maintain proper sarcomere form, tight junctions and have functional expression of ion channels,
supporting that the defects are associated with basement membrane alone. Mice that develop a functional heart will eventually die from cephalic defects. This phenomena first appeared quite strange given that perlecan is not expressed in the central nervous system (Handler et al., 1997). Exencephaly usually occurs from improper neuronal tube closure, however, perlecan-null mice have a normal neural tube and proper closure of the neuropores (Arikawa-Hirasawa et al., 1999; Costell, 1999). Under normal conditions there is a solid layer of ectodermal cells encompassing the brain tissue, but in the absence of perlecan, small clefts are formed about 20-30 µm wide. Cephalic mesenchyme move through the clefts and invade the ectoderm layer. The barrier separating the brain tissue from ectoderm is disrupted allowing the brain to fuse with the neighboring ectoderm. The laminar architecture surrounding the brain is severely distorted indicated the basement membrane barrier is lost (Costell, 1999). The embryos also develop holes in their fore- and midbrain and have collapsed brain vesicles. Perlecan-null mice also experience severe bleeding within several tissues, such as the lung, skin and brain, caused by weakening of the blood vessel wall.

Figure 1.9 shows the protein and mRNA expression of perlecan at the epiphyseal plate, where it is required for proper chondrocyte organization and bone mineralization. Reduced levels of perlecan in mice, mimicking the SJS phenotype, lead to failure of the chondro-osseous junction of developing bones, as shown in figure 1’10 (Arikawa-Hirasawa et al., 1999). The reduction of perlecan interferes with normal growth plate organization, causing bones to become shorter, thicker and misshapen. This is commonly seen in long bones, sternum, and innominate bone. Under normal circumstances, chondrocytes form columnar structures of cell layers (i.e. resting, proliferative, pre-hypertrophic and hypertrophic zones), but when perlecan expression is reduced
these zones become highly disorganized and expand (Arikawa-Hirasawa et al., 1999; Costell, 1999). The COJ is lost and chondrocytes from the perichondrium layer invade the surrounding tissue and generate ectopic ossification (Costell, 1999). The matrix of the perichondrium layer otherwise maintains normal levels of glycosaminoglycans and aggrecan, and maintains proper organization of collagen and fibronectin fibrils, yet overall tissue architecture is lost (Arikawa-Hirasawa et al., 2001). The catabolic turnover of perlecan’s HS chains by proteases, such as heparanase, at the COJ supports VEGF signaling and promotes angiogenesis for cartilage matrix remodeling and bone formation (Brown et al., 2008; Ishijima et al., 2012). At hypertrophic zone of the growth plate, intact perlecan containing GAGs chains, functions acts as barrier separating the mineralized bone tissue from cartilage.
Figure 1.9 Perlecan localization in growth plate of developing long bone. Perlecan protein and mRNA expression in developing hind limb. Perlecan (red staining in panels A and C) creates a barrier at the chondro-osseous junction separating mineralized bone and vascularized bone marrow from avascular cartilage. Shown in A, perlecan is concentrated in the hypertrophic zone (Hz) of the growth plate of a D4 mouse. Bone matrix is shown in brown below the Hz and growing cartilage at the top of the figure is seen by nuclear stain (green). Panel B shows strong perlecan mRNA expression (dark) on embryonic day 13 in pre-hypertrophic avascular cartilage and in the periosteum lining periphery of bone. Similarly, perlecan protein is detected in the Hz regions of developing mice (D18) and pre-hypertrophic avascular cartilage.
Figure 1’10 Loss of perlecan secretion impacts growth plate organization. Type X collagen (green; left panels) and Von Kossa stain (right panels) show disruption of the Hz in perlecan mutant (Pln mut.) mice compared to wild-type (WT). Image taken from Lowe et al. (2014). Arrows indication changes in the hypertrophic region.
Perlecan deficient mice develop brittle bone due to changes in bone elastic modulus, mineral density, and cortical bone thickness (Lowe et al., 2014). Poor bone quality in perlecan deficient mice results from the disruption of the lacunar-canalicular system (LCS) of mineralized bone, where perlecan is part of the PCM surrounding the osteocytic processes that preserve fluid flow throughout bone tissue (Thompson et al., 2011). When perlecan levels are reduced there is encroachment of the canalicular wall and decreased pericellular space; yet another barrier function of perlecan, as it prevents mineral formation. This in turn affects solute transport through the LCS. Disrupting the normal fluid flow pattern impacts the amount of drag force and shear stress experienced by osteocytes, and thus forces bone to adapt differently (Wang et al., 2014). Perlecan is thought to function as an osteocyte sensing tether, transmitting extracellular fluid flow drag forces to the osteocyte cell surface (Lowe et al., 2014; Thompson et al., 2011; Wang et al., 2014). The abnormal bone loading response observed in perlecan deficient mice best supports this claim (Lowe et al., 2014; Wang et al., 2014). Similarly, perlecan is involved in maintaining muscle composition and mass under loaded/unloaded conditions (Ning et al., 2015; Xu et al., 2010).
2.0 Chapter 2

As published in the Journal of Matrix Biology\textsuperscript{1}

Single Molecule Force Measurements of Perlecan/HSPG2: A Key Component of the Osteocyte Pericellular Matrix

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2.1 Abstract

Perlecan/HSPG2, a large, monomeric heparan sulfate proteoglycan (HSPG), is a key component of the lacunar canalicular system (LCS) of cortical bone, where it is part of the mechanosensing pericellular matrix (PCM) surrounding the osteocytic processes and serves as a tethering element that connects the osteocyte cell body to the bone matrix. Within the pericellular space surrounding the osteocyte cell body, perlecan can experience physiological fluid flow drag force and in that capacity function as a sensor to relay external stimuli to the osteocyte cell membrane. We previously showed a reduction in perlecan secretion alters the PCM fiber composition and interferes with bone’s response to mechanical loading in vivo. To test our hypothesis that perlecan core protein can sustain tensile forces without unfolding under physiological loading conditions, atomic force microscopy (AFM) was used to capture images of perlecan monomers at nanoscale resolution and to perform single molecule force measurement (SMFMs). We found that the core protein of purified full-length human perlecan is of suitable size to span the pericellular space of the LCS, with a measured end-to-end length of $170 \pm 20$ nm and a diameter of 2-4 nm. Force pulling revealed a strong protein core that can withstand over 100 pN of tension well over the drag forces that are estimated to be exerted on the individual osteocyte tethers. Data fitting with an extensible worm-like chain model showed that the perlecan protein core has a mean elastic constant of 890 pN and a corresponding Young’s modulus of 71 MPa. We conclude perlecan has physical properties that would allow it to act as a strong but elastic tether in the LCS.
2.2 Introduction

The basement membrane (BM) protein perlecan (Iozzo and Schaefer, 2015), also known as heparan sulfate proteoglycan 2 (HSPG2), is one of the largest (470-700kDa) and oldest (>550 million years) matrix molecules with many important functions (Farach-Carson et al., 2014a; Iozzo et al., 1994). One of the ancient key functions of perlecan is to maintain the integrity of functional borders separating tissues and tissue layers through its long (~200 nm) (Farach-Carson and Carson, 2007) and five modular core protein domains that interact with various matrix molecules (Farach-Carson et al., 2014a). Perlecan’s modular structure (figure 2.1) has been retained throughout millions of years of evolution (Farach-Carson et al., 2014a; Warren et al., 2015), and the largest portion of perlecan, domain IV, is about 90% identical across mammalian species (Farach-Carson et al., 2014a). The perlecan gene, HSPG2, can be found in early simple metazoan organisms with few cell layers and in developing and adult tissue borders in mammals as well. These tissue borders represent functional barriers such as those between extracellular matrix (ECM) and blood (e.g., glomerular basement membrane (BM) (Kanwar et al., 1984), among muscle fibers (Rogalski et al., 2001), and at the osteochondral junctions of long bones (Brown et al., 2008). Perlecan contributes to the structural stability of BM by binding to and linking together the individual laminin and collagen networks (Costell, 1999), which is critical for kidney’s filtration function. Although initially identified as a structural component in BM, regulatory functions of perlecan have been recognized through the interactions of its attached heparan sulfate glycosaminoglycan (GAG) side chains with growth factors and other matrix proteins (Farach-Carson and Carson, 2007). This newly gained evolutionary function for perlecan is especially prominent in tissues
that typically experience mechanical loading, such as muscle, cartilage and bone tissue. In skeletal muscle tissue, perlecan maintains fast muscle mass and fiber composition in response to overloading (Xu et al., 2010). In perlecan deficient slow-twitch soleus muscles, unloading resulted in loss of muscle mass through autophagy by the mTORC1 pathway (Ning et al., 2015). Recent studies demonstrated that the perlecan-rich pericellular matrix (PCM) surrounding chondrocytes in articular cartilage exhibits distinct mechanical properties from the territorial ECM and modulates chondrocytes responses to mechanical stimuli via binding and release of FGF-2 (Prein et al., 2015; Vincent et al., 2007; Wilusz et al., 2014). More recently, our group identified the presence of perlecan in another functional interface, i.e., osteocyte lacunar-canalicular system (LCS) and its involvement in osteocyte-mediated mechanosensing in response to load-induced fluid flow (Thompson et al., 2011; Wang et al., 2014).
Figure 2.1 Schematic representation of the domain organization of human perlecan. Five structural domains are depicted as a strand of individually folded modules that share homology to various protein families. The entire molecule is 4391 amino acids with a core molecular mass approximately 470 kDa. The core protein, excluding the GAG side chains, is predicted to span 100-200 nm in length. With the addition of GAG chains (each 10–100 kDa or 20-150 nm) in domains I and V the molecule’s dimensions can be extended.
Interstitial fluid flow in the bone LCS functions as a major mechanical stimulus that drives various cellular processes during bone adaptation (Fritton and Weinbaum, 2009). Being the most abundant bone cells and strategically positioned in the middle of mineralized matrix, osteocytes serve as the primary sensors to perceive external mechanical signals through interstitial fluid flow (Burger et al., 1995; Weinbaum et al., 1994). For the past two decades, advances from in vitro and in vivo studies have elucidated multiple mechanotransduction pathways (e.g., Wnt/SOST and OPG/RANKL) for osteocytes to regulate the functions of osteoblasts and osteoclasts and thus orchestrate bone’s response to mechanical stimuli (see recent reviews (Bonewald, 2011; Dallas et al., 2013; Klein-Nulend et al., 2013)). However, the mechanosensing apparatus that allows osteocyte to detect interstitial fluid remained unclear. Although a fibrous PCM containing proteoglycans and transverse tethers were hypothesized to surround the osteocytes in the bone LCS by Weinbaum and coworkers in (Weinbaum et al., 1994; You et al., 2001), respectively, the chemical composition of the PCM and the tethering candidates were not identified until ten years later. In 2011, we confirmed perlecan to be an important component of the osteocyte PCM, where reduced expression of perlecan results in fewer tethering elements within the pericellular space and narrower canalicular channels (Thompson et al., 2011). Furthermore, we demonstrated a 30% reduction of the PCM fiber density and the lack of anabolic responses to in vivo mechanical loading using a perlecan deficient mouse (Wang et al., 2014). Based on these in vivo results and the known properties of perlecan, we hypothesized that the perlecan-containing PCM tethers serve as flow sensors in the bone LCS and the fluid drag forces experienced by the PCM tethers were predicted to be at piconewton levels under physiological loading conditions.
The question remains as to whether perlecan molecule can withstand the predicted fluid drag forces in the bone LCS.

Although recognized as a key structural component of many territorial and pericellular matrices, perlecan’s mechanical properties have yet to be explored. Given perlecan’s linear modular structure, we considered it an ideal candidate for single molecule force measurements (SMFMs) using atomic force microscopy (AFM). This method has been widely adopted to study the mechanical strengths of individual proteins, ligand-receptor interactions, and large protein complexes (Neuman and Nagy, 2008). Selective examples include investigations of mechanical properties of proteins involved in cytoskeleton rearrangement (Harris et al., 2007; Marszalek et al., 1999; Rief, 1997; Wijeratne et al., 2013), tissue elasticity and ECM integrity (Liu et al., 2007; Marin et al., 2003). In particular, SMFM by AFM has been very useful in elucidating the mechanical behaviors of large modular proteins containing tandem repeating motifs including a long stretch of contiguous immunoglobulin (Ig) modules such as titin (Kellermayer, 1997; Marszalek et al., 1999; Rief, 1997). The ability of titin to resist stretching and/or bending under mechanical force was demonstrated using SMFMs (Li et al., 2000; Wang, 2001).

Perlecan contains 22 Ig repeats in domain I, similar to titin, but the abilities of perlecan to provide mechanical stability and flexibility to perlecan-rich tissues and their borders have not been measured.

Our work here sought to investigate perlecan’s mechanical properties to understand how perlecan might serve as an osteocyte PCM mechanosensing tether that endures physiological fluid flow drag within the bone LCS. We hypothesized that the perlecan core protein sustains tensile forces without unfolding under physiological
loading conditions. For these studies, we carried out AFM imaging and force measurements on purified full-length perlecan with and without its GAG sidechains. While perlecan was engaged with the AFM tip and the tip was moved away under a controlled fashion, we recorded the tensile force experienced by perlecan as a function of extension length. The force-extension curves were fit with a worm-like chain model to determine perlecan’s mechanical properties. Our objective was to demonstrate that the physical properties of perlecan are consistent with its proposed mechanosensing role in the bone LCS.

2.3 Materials and Methods

2.3.1 Perlecan purification from HT-29 conditioned media

HT-29, a human colon carcinoma cell line, formerly called WiDr, was chosen because these cells produce perlecan as their primary HSPG (Chen et al., 1987; Iozzo, 1984). Cells were purchased from the American Type Culture Collection (Cat. No. CCL-218, ATCC, Manassas, VA) and cultured as described previously (Grindel et al., 2014; Iozzo, 1984). When cells were nearly 100% confluent as judged by eye in Corning Hyperflasks, they were switched to 2% (v/v) fetal bovine serum (FBS) medium, and medium was collected and processed with protease inhibitors as recently described (Grindel et al., 2014). The solutions were processed through a S34100 Amicon spiral wound 100 kDa molecular weight cutoff media concentrator cartridge. The resulting high molecular weight concentrated solution was subjected to diethyl aminoethyl (DEAE) anion exchange chromatography (GE Healthcare, Pittsburgh, PA). Equilibration buffer contained 2 M urea, 50 mM PIPES (pH 7.0), 250 mM NaCl, 2.5 mM EDTA, 0.5 mM benzamidine, 0.5 mM phenylmethyl sulfonylfluoride (PMSF), and 0.02% (w/v) sodium
azide. Elution buffer was identical to equilibration buffer except the NaCl was 750 mM. Eluted 1.5 mL fractions were collected and the absorbance at 280 nm read. Fractions were individually analyzed by dot blot immunoassay with anti-perlecan domain IV antibody A7LC (described below). Pooled fractions containing perlecan were dialyzed in MilliQ water and centrifuged in a speed vac until the desired concentration was obtained. The resulting perlecan enriched solution was separated by Sepharose CL-4B (Sigma-Aldrich, St. Louis, MO) gel filtration chromatography in the presence of 0.8 M NaCl phosphate buffered saline (PBS, pH 7.4) buffer. Perlecan peak containing fractions were pooled dialyzed in MilliQ water and centrifuged in a speed vac. Perlecan pools were subjected to gradient heparin Sepharose 6 fast flow (GE Healthcare) chromatography in PBS containing 0.2, 0.3, 0.5, 1.0, and 1.5 M NaCl. Perlecan, as determined by A7LC dot blot, eluted at 0.3 M NaCl. Perlecan was centrifuged in a speed vac and dialyzed in a final PBS buffer. Samples were aliquoted and stored at -80°C. Purity was assessed by silver stain as described below.

2.3.2 Cleavage of glycosaminoglycan (GAG) side chains

To investigate the elastic properties of the core protein exclusively, perlecan’s GAG side chains were cleaved by incubating the molecule with heparitinase I, II, III and chondroitinase ABC in PBS with 2 mM calcium chloride at 0.1 units per enzyme at 37°C overnight. Enzymes were separated from the core protein by passing samples through an anti-perlecan A74 antibody (a generous gift from John M. Whitelock) affinity column following established protocols (Marszalek et al., 1999). All final perlecan containing fractions were pooled, dialyzed in MilliQ water and centrifuged in a speed vac. The A7L6C (Sigma-Aldrich) antibody was used for all dot-blot and western blot analyses.
Purity and removal of GAG chains was confirmed by collapse seen via silver stain. Heparan sulfate from bovine kidney was purchased from Sigma-Aldrich and used as a control for AFM pulling.

2.3.3 Dot blot and silver stain conditions

Purified full-length and GAG digested perlecan were denatured at 99°C with reducing sample buffer (final 60 mM Tris-HCl, 1% (w/v) SDS, 10% glycerol, 2% (v/v) 2-mercaptoethanol, pH 6.8 with tracking dye) for 5–10 min. Samples were separated with SDS-polyacrylamide gel electrophoresis (PAGE) in 1 mm Novex NuPAGE 4–12% acrylamide gradient Bis-Tris buffered gels (Invitrogen, Carlsbad, CA) in a Novex NuPAGE system (Invitrogen) with 1 × MOPS SDS buffer (Invitrogen) at 150 constant volts for approximately 80 min. SeeBlue® Plus2 molecular weight marker (Invitrogen) was used to determine molecular weight. Silverstains were performed with the Silverquest Silver Stain kit according to manufacturer's directions (Invitrogen). For perlecan dot blots, 20 µL of sample was added to a Bio-Rad slot blot apparatus (Cat. No. 170-6545) and allowed to bind pre-PBS rinsed 0.45 µm nitrocellulose membrane for 2 h at room temperature. After vacuum suction, the dot blot was blocked in a 3% (w/v) BSA Tris buffered saline with 0.05% (v/v) Tween-20 (TBST) for 2 h at room temperature. A7LC antibody (Invitrogen) at 1:5,000 was incubated in the blocking solution overnight on a 4°C shaker. After 3 by 5 min TBST washes, the membranes were incubated with 1:200,000 goat anti-rat HRP conjugated antibody in 3% BSA TBST for 2 h at room temperature. Blots were washed again and incubated with chemiluminescence substrate (West Dura Extended Substrate, Pierce, Rockford, IL) for 5 min before being exposed to film.
2.3.4 AFM experiments

For all AFM studies, data was gathered on a Nanoscope V with Multimode 8 AFM (Bruker AXS, Santa Barbara, CA). The spring constant of each cantilever was calibrated prior to each experimental run according to the vendor’s instructions. The cantilever deflection-sensitivity was calculated from a force curve generated by pressing the tip against the gold substrate in either air or PBS solution. Once sensitivity was determined, the thermal tune method was used to calculate the cantilever spring constant. For this method, the tip retracted from the substrate surface and oscillated to identify the correct resonance frequency.

For imaging experiments, discs of mica were glued to steel discs with epoxy and allowed to dry overnight. The dried mica disks were cleaved with Scotch tape to expose a fresh surface. 10 µL of perlecan samples (10 µg/mL) were left on mica for 5 min, washed with double distilled water and then dried with nitrogen air. Imaging was carried out in ScanAsyst mode at room temperature using ScanAsyst-Air tips (spring constant: 0.4 N/m and radius: 2 nm, Bruker AXS). A total of 27 AFM images of full-length perlecan were traced using ImageJ (National Institutes of Health, Bethesda, MD) (Schneider et al., 2012) and Engage digitizer software (Free Software Foundation). A program written in MATLAB (The Math Works, Natick, MA) was used to interpolate the xy coordinates of the perlecan contours and calculate the persistence length ($L_p$) of perlecan using a tangent to tangent correlation function (Wiggins et al., 2006),

$$\langle \cos(\theta) \rangle = \exp(-L / 2L_p)$$  \ (Equation 2.1).

The $L_p$ is a measure of bending stiffness of a polymer or linear molecule, with a high $L_p$ conferring low flexibility. $L_p$ is defined as the distance over which the correlation of
tangents is lost, among two points along the contour trace. The angle, $\theta$ is that between two tangent vectors at a pair of points separated by a given length ($L$).

Pulling experiments were performed in solution under contact mode using a calibrated MLCT-D tips (spring constant: 0.01 N/m and radius: 20 nm, Bruker AXS). MLCT probes have an intentionally dull tip ideal for force measurements and imaging extremely delicate samples in fluid. Perlecan at 20 nM in PBS was incubated at 37°C prior to being deposited onto a fresh gold substrate at room temperature for 10–15 min. The AFM tip was brought in contact with the surface for 1–3 s to allow the perlecan molecule to attach to the silicon nitride cantilever tip. All of the force measurements were obtained in aqueous PBS (pH 7.4) with a pulling velocity of 1000 nm/s. Aggregation of perlecan molecules was minimized by using a low protein sample concentration as previously described (Chen and Hansma, 2000). At this diluted concentration (one perlecan molecule per 0.8 µm$^3$), the fluid volume displaced by the tip (radius of 20 nm) moving 10–100 µm into the fluid was anticipated to be approximately 0.01–0.1 µm$^3$. Thus, it was unlikely to have multiple perlecan molecules attached to the tip and the recorded forces were most likely due to single molecule engagements.

For module unfolding experiments, 1 µL of 10 mM of dithiothreitol (DTT) was added to 10 µL of perlecan solution and incubated as described above, and force measurements were taken in 1 mM of PBS and DTT buffer solution. Each experiment lasted from 1–3 h.

To obtain successful tip-sample engagement, for each experimental condition roughly 500-3000 force measurements were performed. In total, 54 native full-length, 57 GAG digested, and 345 DTT treated perlecan force curves were collected for data
analysis. Pulling events capturing multiple tip interactions or oligomers were excluded from data analysis by selecting curves with only one detachment peak and of extension length similar to a single perlecan monomer. The force curves were fitted with either the extensible WLC (eWLC) model (Bustamante et al., 1994; Calderon et al., 2009) (for native perlecan with or without GAGs),

$$x = L_c \left[ 1 - \frac{1}{(4\beta L_p F)^{1/2}} + \frac{F}{K} \right], \text{ (equation 2.2)}$$

or inextensible WLC model (Bakajin et al., 1998; Bustamante et al., 1994; Chen et al., 2010; Ono et al., 2003) (for denatured DTT treated perlecan),

$$F(x) = \frac{1}{L_p \beta} \left[ \frac{1}{4(1 - x/L_c)^2} + \frac{x}{L_c} - \frac{1}{4} \right], \text{ (equation 2.3)}$$

where \(x\) is the extension, \(F\) is the force, \(K\) is the elastic stretch modulus and \(\beta = 1/k_BT\) where \(k_B\) is the Boltzmann constant, \(T\) is the temperature, \(L_c\) is the contour length of the total end-to-end length of an extended molecule and \(L_p\) is the persistence length. Histograms of the \(L_c\) and \(L_p\) distributions were fitted to a Gaussian curve to get statistical measures of the two parameters. For unfolding studies, peak-to-peak distance was measured as change in contour length (\(\Delta L_c\)), which reflects the size of a particular region of the protein core that is unfolding. Equation 2 (eWLC) was used to model conditions where the protein remain folded, whereas, the inextensible WLC model (Eq. 3) is more suitable for protein unfolding and therefore used for denatured conditions.
2.4.0 Results

2.4.1 Perlecan morphology by AFM imaging

Purified perlecan was isolated from the conditioned media of HT29 cells, a human colon carcinoma cell line, following previously established protocols (Whitelock et al., 1999) (figure 2.1 A). AFM images showed single isolated perlecan monomer structures (figure 2.1 B). Although protein aggregates were observed on occasion, single monomers were clearly visible. The core protein contained globular features having diameters of 2–4 nm and heights of 5–10 nm.
Figure 2.1 Three-dimensional AFM images of single perlecan molecules. (A) Lane 1 is a Coomassie stain and lane 2 is a perlecan western blot (domain IV antibody 3135) of purified perlecan. (B) Single molecules exhibited a beads-on-a-string appearance with an extended linear-shaped conformation. Globular features were observed, possibly corresponding to protein modules. Perlecan molecules were measured to have diameters of 2–4 nm and heights of 5–10 nm.
Single linear molecules were measured from end-to-end to determine a contour length (figure 2.3 A). The perlecan contour was converted to digitized coordinates to obtain accurate measurements (figure 2.3 B). A distribution of perlecan contours revealed a most common extension of ~170 ± 20 nm in length (figure 2.3 C). Persistence length was determined by plotting the cosine correlation function against the contour separation (figure 2.3 D). A high correlation (~1) exists when two different coordinates have tangent vectors with similar angles, as one would expect in a straight or rigid molecule. On the other hand, when the tangents are pointing in opposite directions, as occurs in a curved region (figure 2.3 B), the correlation is lower (< 1). The plot in figure 2.3 D shows that the cosine correlation for perlecan decreases the further away two points are from one another. Overall, perlecan molecules favored a long thin flexible conformation with a $L_p = 19 \text{ nm} \pm 5 \text{ nm}$ (Eq. 2.1). Image analysis was attributed to the perlecan core itself, because GAGs were not consistently visible under the conditions used to optimally image core protein structures.
Figure 2.3 Analysis of the contour and persistent lengths of perlecain. (A) A representative AFM image of full-length perlecain used in the analysis (total 27 images). (B) An enlarged zoom-in image of the box in (A), showing the digitized contour tracing of the molecule used to find the contour length, $L_c$ (C). Histogram showing the distribution of the $L_c$ is fitted to a Gaussian curve (solid line); and the peak of the Gaussian is 170 nm with a standard deviation of 20 nm. (D) Cross-correlation function $\langle \cos(\theta) \rangle$ as a function of contour separation. The data were fitted to Eq. 1 to obtain persistence length $L_p = 19$ nm (as the $\langle \cos(\theta) \rangle = e^{-1/2} = 0.6$).
2.4.2 Perlecan Elastic Properties by AFM Pulling

The AFM pulling scheme we used is depicted in figure 2.4 A. While perlecan molecules remained attached to the gold substrate, randomly chosen segments of the molecule, which could be either core protein or GAG, were located then pulled upon using the AFM tip and extended until the sample detached from the cantilever. The resulting force-extension curves of the full-length native perlecan showed a single detachment peak with no noticeable unfolding events (figure 2.4 B) regardless of where the tip attached to the proteoglycan. For SMFMs, force curves follow a similar trace pattern, where the last peak represents the rupture of the tip-sample interaction. This detachment peak is distinguished from peaks that represent unfolding processes, in that after the sample-tip interaction is lost the tip snaps back to its starting neutral position and thus the force returns to baseline levels. We fit the force-extension curves to the eWLC model, which predicts perlecan is extended beyond its resting contour length, given the relative high forces (~150 pN, figure 2.4 B).
Figure 2.4 Stretching native full-length perlecain. (A) Purified perlecain was adsorbed onto a gold substrate and brought into contact with an AFM tip. As the stage moved away from the tip the perlecain molecule was pulled while force was recorded. (B) Two representative force-extension curves of pulling events of native perlecain and the GAG digested form with eWLC fitting in solid lines. The measured extension values in the force-extension curves ranged from 40–445 nm for native perlecain and 23–279 nm for the perlecain core. For both native perlecain and the GAG digested form there was only one peak observed. No unfolding occurred in either case.
The most probable values, resulting from Gaussian fits to histogram distributions, are $L_p = 1.2$ nm, and $K = 2000$ pN (figure 2.5 A and B). To examine the core protein exclusively and ensure the measurements were not associated with the GAG portion of the proteoglycan, we completely removed the GAGs by digesting perlecan with heparitinase I, II, III and chondroitinase ABC and repeated the pulling analysis. We found that the profile of the force curves resembled what we saw in full-length perlecan, displaying a single detachment. When we fit the data to the eWLC model, we observed the core protein of perlecan to have $L_p = 22$ nm and $K = 890$ pN (figure 2.5 C and D). The $L_p = 22$ nm of the perlecan core from pulling is consistent with the $L_p = 19$ nm obtained from imaging. However, full-length perlecan had a $L_p = 1.2$, much lower than that of the core.
Figure 2.5 Elasticity of full-length perlecan with and without its GAG side chains. Histograms showing (A) native perlecan having a $L_p$ of 1.2 nm and (B) a $K$ of 2000 pN from fitting to a Gaussian distribution. Digesting away the GAG chains increased perlecan’s $L_p$ to 22 nm (C) and decreased $K$ value (890 pN) (D).
In the experiments above, no module unfolding with the characteristic sequence of multiple peaks was observed, perhaps due to the presence of intradomain disulfide bonds. To confirm this, we added DTT to break the disulfide bonds of perlecan, to enable the force-induced unfolding. When 1 mM DTT was added, the force-extension curves exhibited sequences of sawtooth pattern, characteristic of the sequential unfolding of the protein modules (Oberhauser et al., 1998; Rief, 1997; Rief et al., 1999) (figure 2.6 A). The force curves were fitted with an inextensible WLC model (Eq. 3). Figure 2.6 B shows that from the Gaussian fits to the histogram, the unfolding force with the highest occurrence frequency was 110 pN. The histogram of the change in contour length $\Delta L_c$, has a peak at 35 nm (figure 2.6 C). The observed unfolding forces of disulfide-cleaved perlecan are within the range of forces required to unfold elastic behaving proteins (Oberhauser et al., 1998; Rief, 1997; Rief et al., 1999). Under normal circumstances, perlecan exists as a stable intact protein, and only when chemically denatured by oxidizing the disulfides did perlecan unfold under tensile mechanical stress.
Figure 2.6 Perlecan was unfold only after being pretreated with a denaturing agent. (A) In the presence of DTT, multiple unfolding peaks were observed. Large initial peaks are due to non-specific attraction forces that cause tip to snap-in to the surface and therefore are excluded from data analysis. These peaks were fitted to the inextensible WLC model (dashed lines) to obtain the unfolding force. Note that the last peaks were usually as high as 500 pN. (B) The histogram of the unfolding force, fitted with a Gaussian distribution (solid line), shows a peak at 110 pN. (C) The histogram of the change in contour length between the unfolded peaks, \(\Delta L_c\), shows a peak at 35 nm.
2.5 Discussion

Our AFM data revealed that perlecan has a long and strong core protein that resists conformational changes when under mechanical distress. This result supports our previous hypothesis that perlecan possesses the physical properties to serve as a transverse tether in the LCS to sense fluid drag caused by mechanical loading (Wang et al., 2014). We measured the unstressed end-to-end length of the perlecan core protein (~170 nm) to demonstrate that perlecan was of sufficient size to transverse the annular gap (~100 nm) and tether the osteocyte cell body to the canalicular wall (You et al., 2004). In addition, we demonstrated the mechanical strength of perlecan core protein through single molecule pulling. Compared to the stretch modulus of the elastic protein titin (185 pN) (Linke et al., 1998), the stretch modulus K of perlecan core protein was found to be almost 5 times greater (890 pN). Assuming the perlecan core protein as a rod with a cross-sectional area A (diameter of 4nm, figure 2.2), the tensile modulus of the core protein $E = K/A = 71$ MPa, which are smaller than those of collagen I fibril (< 5 GPa, (Hofmann et al., 1984)) and actin filaments (1.8 GPa, (Kojima et al., 1994)) but much larger than that of focal adhesion (5.5 KPa, (Balaban et al., 2001)).

For perlecan to function as a viable osteocyte cell tether, it must be able to traverse the pericellular space and endure fluid flow drag forces. AFM images were taken to obtain an accurate measure of the true length native purified monomeric perlecan. Previously measurements of various aspects of perlecan’s dimensions and molecular topography have been reported, including one study finding the core to be around 80 nm in length and each GAG attachment to extend up to 170 nm (Saku and Furthmayr, 1989). In another study, AFM imaging was used to characterize the size of perlecan, revealing two
molecular volumes of the protein core at 290 nm$^3$ and 710 nm$^3$ (Chen and Hansma, 2000). Our results differ from previous studies, possibly due to the difference in the source perlecan and isolation method. We found on average, measured by AFM images, that perlecan extended about 170 nm in length (figure 2.2), agreeing with our past prediction based upon composite of detailed rotary shadowing electron microscopy and AFM images of recombinant domains (Farach-Carson and Carson, 2007). Variations can be attributed to perlecan’s flexible nature and ability to fold onto itself in variety of ways. In addition, it must be considered that adsorbed proteins can undertake substrate-induced conformations. Despite these uncertainties, our measurements of “resting” linear dimensions lead us to conclude that a single perlecan monomer easily can span the entire pericellular space, which has been measured to be about 100 nm wide, from osteocyte cell body to the mineralized bone surface (Lai et al., 2015).

To determine if perlecan is strong enough to handle mechanical loading, we estimated the fluid drag forces under normal loading conditions (400 microstrain in mouse tibia) to be 18.4 pN over one micron length of cell process (Wang et al., 2014). This translates to less than 1 pN of drag force acting transversely on each tether and less than 10 pN tension along the long axis of the core protein, assuming the tether anchors at the bone wall and cell membrane at an angle larger than 2.8 degree to the radial direction of the canaliculus. This anchoring angle can be easily achieved if the effective length of the tether is no shorter than 100.04 nm to span the 100 nm gap between the cell membrane and the wall (Fig. 7). Our data suggest that perlecan is strong enough to sustain the tensional forces under physiological loading conditions (~10 pN for 400 microstrain loading), given its much higher rupture forces (~150 pN, figure 2.4) under AFM pulling.
The high end of physiology strain is about 2000 microstrain, resulting 5 times of the fluid drag, which is still lower than the rupture force measured for the perlecan core protein. In the pulling experiments, it was impossible to control the exact locations that the perlecan monomer anchored to the AFM tip and the substrate. Thus, the final rupture might occur at either end of the contact regions or along perlecan’s linear structure including protein core and GAG side chain. However, unfolding or rupturing the protein core was highly unlikely under the testing condition. We did not observe the sequential peaks that are characteristic of protein unfolding and we only observed single peaks as the full-length perlecan was pulled (figure 2.4). Protein core unfolding could be observed after treatment with DTT to break the disulfide bonds (figure 2.6). Even under this denatured condition, the mean force to unfold perlecan was at the level of 110 pN and the final rupture forces could be as high as more than 500 pN (see the last peaks in figure 2.A). These data led us to conclude that the perlecan protein core can easily sustain tensions at the order of 10 pN under physiological loading conditions of the bone LCS.
Figure 2.7 Perlecan tether under tension caused by fluid flow in the bone LCS. (A) A TEM image showing the transverse tethers span the space (~100 nm) between cell process membrane and canalicular wall. (B) The fluid drag force ($F_d = \sim 1$ pN) along the axial direction induces a tension ($T = \sim 10$ pN) within the tethering fiber, assuming the tether anchors at a small angle ($\theta = 2.87^\circ$) as suggested in (A). The projection of the tension forces balances with the drag force ($2T \sin(\theta) = F_d$). The tension in the tether depends on the fluid drag force and the anchoring angle. Larger angles are anticipated to be associated with smaller tension forces under a given fluid drag force.
As a potential mechanosensing tether, we must compare the strength of perlecan itself to those of the anchors that it forms with the osteocyte cell membrane and bone matrix. One possibility is that perlecan interacts with the mineralized bone via its GAG side chains of domain I. The bone matrix is rich in collagen I, which is involved in heparin binding. It has been shown that both soluble and cell surface heparan sulfate proteoglycans bind with high affinity to native interstitial collagen I fibrils, with Kds approximately $10^{-10}$ M and $10^{-9}$ M, respectively (Koda and Bernfield, 1984; Koda et al., 1985). Our lab has previously shown that perlecan domain I can bind directly to collagen I monomers and fibrils, via its heparan sulfate side chains (Yang et al., 2005). The cleavage of perlecan’s domain I GAG chains with heparinases, but not chondroitinase, completely prevented binding to collagen I (Yang et al., 2005). Single molecule approaches have been used to measure the binding strength of heparan sulfate, with one study finding that as high as 310 pN was required to rupture a multivalent heparan sulfate-ligand interaction (Guo et al., 2015). This high strength of GAG-ligand interaction may explain our observations that perlecan with GAG showed a shorter persistent length $L_p$ (1.2 nm vs. 22 nm) and a higher stretch elasticity $K$ (2000 vs. 890 pN) than perlecan devoid of GAG (figure 2.5). It has been shown that perlecan links together the laminin and collagen IV rich networks of the epidermal BM in a spot-weld-like manner (Behrens et al., 2012). Perlecan’s heparan sulfate chains are integrated into collagen network, which is likely how perlecan interacts with the mineralized bone wall of the LCS. Separating these two perlecan-linked independent networks requires repeated application of mechanical force. Unfortunately, the total amount of force required to break the perlecan linkage was not determined. In any case, the forces required to
separate the bound protein-protein and protein-carbohydrate pairs (190-400 pN) (Bell, 1978; Bucior et al., 2004; Dammer et al., 1995) tend to be well above the forces exerted by fluid drag in the LCS (10 pN). If indeed domain I is the attachment point to bone surface, it is most likely the opposing C-terminal end of perlecan (domains IV-V) is anchored to the osteocyte cell surface. Domain V interacts with cells surface integrins, most notably β1 integrin (Hayashi, 1992). Interestingly, β1 integrin was shown to be involved in bone mechanotransduction, whereupon the knockout of β1 integrin in cortical osteocytes limited bone loss resulting from disuse (Phillips et al., 2008). Domain IV also serves as a likely link for cell attachment, considering its length (nearly half of the perlecan) and its multiple repeats of Ig motifs (figure 2.1), which are often recognized in cell adhesion processes (Wai Wong et al., 2012). It is also possible that the perlecan tether may be indirectly linked to the osteocyte surface through a PCM-cell membrane complex. Despite the lack of mechanical measurements of the anchors at the two ends of the PCM tether, perlecan’s ability to bind and link together various ECM proteins supports further investigations of the possible tether configurations in the bone LCS.

There are several limitations for our study. In many AFM single molecule studies, small recombinant proteins are often used with a priori known unfolding peak patterns. With perlecan, the force-extension profile was unknown, raising the concern whether or not our AFM pulling scheme was capable of detecting or causing any unfolding events. We thus destabilized the disulfide bonds of perlecan, making it more vulnerable to force-induce unfolding (figure 2.6). This set of experiments increased our confidence that the lack of multiple peaks during pulling perlecan and perlecan core protein indicates that there was no protein unfolding under the testing condition. Another limitation was that
we were unable to control the exact probe attachment points during the pulling experiments. Ideally, we would have the most terminal ends of the protein attached to substrate and AFM tip. This would allow us to capture the stretch profile of the entire molecule. Functionalized substrate and AFM tip may help to achieve this idealized testing configuration.

In this study, AFM was used to capture images of perlecans at nanoscale resolution and to perform single molecule force measurements. We found that the core protein of purified full-length human perlecans is of suitable size to span the pericellular space of the LCS, with a measured end-to-end length of $170 \pm 20$ nm and a diameter of 2–4 nm. Force pulling revealed a strong protein core that can withstand over 100 pN of tension, well over the drag forces that are estimated to be exerted on the individual osteocyte tethers. Data fitting with an extensible worm-like chain model showed that the perlecans protein core has a mean elastic constant of 890 pN and a corresponding Young’s modulus of 71 MPa. We conclude perlecans has the physical properties that would allow it to act as a strong but elastic tether in the LCS.
3.0 Chapter 3. Perlecan/HSPG2: Novel Signaling Role in Early Chondrogenesis and Chondro-Osseous Boundary Formation

3.1 Abstract

Perlecan/HSPG2, a large heparan sulfate proteoglycan (HSPG), is essential for the development and maintenance of musculoskeletal tissue including maintaining the distinct border between cartilage and bone. Perlecan is deposited within the avascular pericellular matrix (PCM) surrounding chondrocytes and turns over nearly completely at the chondro-osseous junction (COJ) of developing long bones. Destruction of perlecan at the COJ converts an avascular cartilage compartment into one that permits blood vessel infiltration, allowing osteogenesis to occur. A reduction in perlecan secretion is associated with chondrodysplasia with widespread musculoskeletal and joint defects, including the disruption of epiphyseal cartilage and bone. This work sought to elucidate novel signaling roles of perlecan in endochondral bone formation. To examine the functions of a highly conserved region of the core protein, purified recombinant perlecan subdomains were tested for bioactivity in a murine chondrogenic cell line, ATDC5, which served as a model for early chondrogenesis. These findings showed a region within domain IV of perlecan (PLN IV-3) that has anti-adhesive properties, promotes cell-cell adhesion, and strongly promotes rapid chondrocyte cell clustering. These properties were lost when a mutation (R3452Q), associated with the human skeletal disorder Schwartz-Jampel Syndrome (SJS), was introduced into the perlecan sequence. PLN IV-3 activity was enhanced when thermally unfolded, likely attributed to increased exposure of the active site. PLN IV-3-induced cell-substratum detachment was accompanied by deactivation of key components of the focal adhesion complex, FAK and SRC. PLN IV-3 suppression of FAK/SRC activity increased pre-cartilage
condensation markers SOX9 and N-cadherin, and also increased cartilage PCM components collagen II and aggrecan. Furthermore, PLN IV-3 reduced signaling through the ERK pathway, where loss of ERK1/2 phosphorylation coincided with reduced FoxM1 protein levels and an increase in transcriptional of cell cycle inhibitors CDKN1C and ATF3. This, in turn, reduced ATDC5 cell proliferation. Together, these findings support the notion that perlecan domain IV is a key force triggering chondrocyte condensation in the developing growth plate, and that loss of such signaling accompanies SJS chondrodysplasias with associated loss of normal COJ boundaries.
3.2 Introduction

The heparan sulfate proteoglycan perlecans play a key role in endochondral bone formation. Reduced perlecan secretion, as seen in human skeletal disorders Schwartz-Jampel syndrome (SJS, OMIM #255800) and dyssegmental dysplasias, Silverman-Handmaker (DDSH, OMIM #224410), leads to widespread skeletal defects with major failure at the epiphyseal plates (Arikawa-Hirasawa et al., 2002, 1999; Ishijima et al., 2012). During embryogenesis perlecan deposition is diffuse throughout the mesenchyme tissue, but becomes prominent in cartilage primordia, particularly at sites where enchondral ossification occurs (French et al., 1999; Handler et al., 1997). As bone is shaped perlecan becomes concentrated at the epiphyseal cartilage ends (Handler et al., 1997). The highest levels of perlecan are seen at the leading edge of bone growth, i.e., the hypertrophic zone of the growth plate and along the COJ (See chapter 1 figure 1.4).

Hence, there are ample opportunities for perlecan to form cell contacts and elicit a chondrogenic response. Chondrogenic activity of perlecan has been linked to the GAG side chains in domain I, through modulating growth factor signaling (French et al., 2002). The complete function(s) of the perlecan core protein, in the context of cartilage biology, remain to be discovered. The evidence suggest perlecan functions as barrier molecule and has an ancient key role in establishing tissue layers (Farach-Carson et al., 2014b; Warren et al., 2015). This is evident at the epiphyseal plate, where it has been shown that the loss of perlecan causes disorganization of the columnar structure of hypertrophic chondrocyte zone (Costell, 1999; Ishijima et al., 2012). The core itself links together the ECM network, by binding to ECM components such as collagen and laminin, and connects cells to their substratum through its ability to bind to integrins (Behrens et al.,
Perlecan’s influence on cell migration is one possible mechanism by which perlecan maintains growth plate organization (Nakamura et al., 2015). The endorepellin/α2β1 integrin interaction disrupts actin stress fibers and focal adhesion, which inhibits endothelial cell migration and has an anti-angiogenic effect (Bix et al. 2004; Bix et al. 2006; (Douglass et al., 2015)Mongiat et al. 2003). Previously our lab identified a peptide within domain IV that promotes cell adhesion, spreading, and cell differentiation (Farach-Carson et al., 2008; Pradhan et al., 2009). More recently we discovered that a region within domain IV supports cell-cell adhesion and prevents cell migration (Grindel et al., 2014). The activity of this domain warrants further investigation.

Pre-cartilage mesenchyme condensation and subsequent chondrocyte differentiation are prerequisites for skeletal formation, however the mechanisms controlling these actions remain unclear. Currently, Bmps, Tgfβ, and Sox9 are considered the key players involved in chondrogenesis, although equally important is the local deposition of territorial matrix. The contacts prechondrogenic mesenchymal cells form with their matrix environment drive key signaling cascades that initiate cytoskeleton reorganization, cell migration, cell-cell adhesion, and cell condensation. These contacts are typically formed by cell surface integrins, α1β1 and α5β1 being key receptors expressed during mesenchyme condensation and differentiation (Djouad et al., 2007; Goessler et al., 2009; Ohno et al., 2002). ECM integrin stimulation leads to activation of focal adhesion kinase (FAK), a molecule that has been shown to be important for precartilage condensation and cartilage nodule formation (Bang et al., 2000; Gemba et al., 2002). FAK activation turns on many downstream signaling cascades including the
mitogen-activate protein kinase (MAPK) pathway (Gemba et al., 2002). MAPK signaling is required at various stages of cartilage development (Bobick and Kulyk, 2008). ERK1/2, one of the principal signaling components of the MAPK cascade, is inactive during the prechondrogenic condensation in developing chick limb (Corson et al., 2003; Kawakami et al., 2003). Here, is suggested MAPK/ERK signaling might play a role in controlling mesenchymal cell proliferation at the distal limb region. An inhibitor targeted to MEK1/2, the upstream activator of ERK1/2, suppressed limb outgrowth (Eblaghie et al., 2003). Inhibition of MEK/ERK leads to decreased expression of cell adhesion molecules (N-cadherin, fibronectin, and α5β1 integrin) important for mesenchyme condensation (Chang et al., 1998; Oh et al., 2000). The extracellular matrix molecule fibronectin is upregulated during chondrogenesis and is one of the key activators of MAPK/ERK signaling leading to chondrogenesis (Thant et al., 2000; Zhao et al., 2006). Chondrogenesis is a highly dynamic process that requires spatial and temporal regulation, therefore, uncovering mechanisms that control MAPK/ERK signaling are of high interest (Corson et al., 2003).

The mesenchyme matrix is rich in proteoglycans such as perlecan. Although recognized as an important molecule for cartilage formation, the exact role of perlecan in prechondrocyte condensation and subsequent differentiation remains unknown. The work described here explored the chondrogenic potential of the perlecan core protein, specially examining the function of domain IV. This region is, surprisingly, the least well-studied despite comprising half the molecule. To examine perlecan domain IV, recombinant subdomains were designed and synthesized for analysis in a chondrogenic cell line ATDC5. The ATDC5 chondrogenic model is outlined in Figure 3.1. ATDC5
cells in standard cultures systems can differentiate into cartilage nodules as occurs during normal development (Newton et al., 2012). Results from this study suggest a perlecan subdomain (PLN IV-3) may control chondrogenesis through MAPK signaling. Activity was assessed by characterizing ATDC5 responses to various perlecan coated substrate and monitoring cell morphology, adhesion, and spreading. The chondrogenic potential of perlecan was investigated by examining the chondrocyte matrix production and gene expression of early chondrogenic markers, Sox9, col21, and aggrecan. Signaling pathways associated with early cell-substrate recognition were examined to determine potential mechanisms of action. Western blot analysis demonstrated PLN IV-3 suppressed FAK/ERK activity. Further investigation of downstream targets supported these findings.
Figure 3.1 ATDC5 in vitro model for precartilage condensation. The ATDC5 cell model has been used extensively to study in vitro precartilage condensation and differentiation. (A) A schematic representation of ATDC5 chondrogenic activity is shown in the top portion of figure. Defining chondrogenic cell features are indicated in yellow. Relative protein levels of collagen 2 (purple) and perlecan (red) are represented by color intensity. (B) For this study, ATDC5 cells were cultured on perlecan coated dishes and assayed for chondrogenic activity as determined by cell morphological, and the expression of proteins and mRNA important for chondrogenesis. Lastly, we sought to elucidate novel signaling mechanism by which perlecan initiated chondrogenesis.
3.3 Materials & Methods

3.3.1 Materials

The following antibodies were purchased from Cell Signaling (Danvers, MA): rabbit anti-phospho-FAK (Tyr397) (catalog no. 3283), rabbit anti-phospho-SRC family (Tyr416) (catalog no. 2101), rabbit phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (catalog no. 9101), and rabbit p44/42 MAPK (ERK1/2) (catalog no. 9102). The mouse anti-FAK clone 4.47 antibody, and chicken anti-GAPDH antibody (catalog no. AB2302) was purchased from EMD Millipore purchased (Darmstadt, Germany; catalog no. 05-537). Rabbit FoxM1 antibody was purchased from Novus Biological (catalog no. NBP1-30961). The rabbit anti-human perlecan genomic antibody 3135 was generated using a proprietary technology developed at Strategic Diagnostics, Inc. (Newark, DE). Antibody 3135 binds to domain IV-3 between amino acids 3295 and 3394. The rat anti-domain IV antibody A7L6 was purchased from Life Technologies (Grand Island, NY). Heparitinase I, II, III, chondroitinase ABC, and were purchased from Sigma-Aldrich (St. Louis, MO). All other materials used were reagent grade or better.

3.3.2 Purification of perlecan and domain IV fragments

Full-length perlecan was purified from HT-29 cells, a human colon carcinoma cell line formerly called WiDr, as previously described (PMID 24833109). Perlecan’s glycosaminoglycan side chains were removed enzymatically using heparitinase I, II, III and chondroitinase ABC (H/C). H/C digested perlecan samples were passaged through an immunoaffinity column immobilized with perlecan domain A74 antibody following established protocols (Whitelock et al., 1999). Domain IV (consisting of 21 Ig repeats encompassing amino acids 1677–3662 and cDNA base pairs 5029–10986 [accession no.
NM_005529.5) was produced as three separate recombinant fragments as previously described (Grindel et al., 2014). IV-1 (aa 1677-2338), IV-2 (aa 2338-3010) and IV-3 (aa 3011-3662) each contained 7 Ig repeats (figure 3.2). The 3D protein structure prediction software Protein Homology/analogY Recognition Engine version 2.0 (PHYRE V2.0; http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) was used to determine protein termini (Kelley and Sternberg, 2009). A domain IV-3 mutant fragment associated with SJS was created by substituting an Arg for Gln at residue 3452 (R3452Q) using site directed mutagenesis (QuikChange II XL kit, Agilent Technologies, Santa Clara, CA).
**Figure 3.2 Perlecan domain IV constructs.** Sequence and structural analysis were used to divide domain IV. In (A), a PHYRE predicted model shows two tandem Ig modules and the linking region where subdomains were separated. Yellow strands indicate beta-sheets and blue regions are alpha helices. Table (B) lists the sequences for each subdomain by Ig modules, amino acid (AA) sequence, and base pair sequence corresponding to accession no. NM_005529.5. (C) Organization of the domain IV constructs are illustrated. IV-1 and IV-2 were cloned into pCEP-pu expression vector, and IV-3 and R3452Q were cloned into a modified Gateway™ system vector. All constructs consists of N-terminal BM40 secretin signal, an enterokinase (EK) cleavage site, and C-terminal 6X His tag. The various restriction sites used for cloning are mapped and indicated by //.
3.3.3 Circular dichroism

Circular dichroic spectra were obtained following previously described protocols (Costell et al., 1997; Hopf et al., 1999). Data was recorded with a Jasco J-815 Spectropolarimeter using the SpectraManager software provided by the manufacturer. Measurements were recorded in the far-UV region using a quartz cell with an optical path length of 1 mm. Temperature interval scan mode was used to profile entire spectra from 20-90°C. Thermal unfolding studies were performed using variable temperature mode at a wavelength of 202 nm. Protein samples diluted to 0.2 mg/ml (2.67 µM) in Milli-Q water. Data was processed with the Jasco spectra analysis software. Secondary structural were predicted using the on-line analysis software DichroWeb (Whitmore and Wallace, 2008, 2004). CD data represents the average value of three separate recordings.

3.3.4 Cell culture

ATDC5 cells, a murine teratocarcinoma-derived prechondrogenic cell line, cultured as previously described by Brown et al. (2008). Cells were cultured in regular growth media Dulbecco’s Modified Eagle’s medium-Ham’s F12 (DMEM-F12; Thermo Fischer Scientific; Waltham, MA), with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and 1X penicillin/streptomycin (Thermo Scientific). Bone marrow derived mesenchymal stem cells isolated as previously described (Thompson et al., 2015), were obtained from Dr. William R. Thompson (Indiana University-Purdue University Indianapolis). Bone marrow derived mesenchyme stem cells were cultured in Iscove’s Modified Dulbecco’s Media (IMDM; Lonza; Alpharetta, GA) with 10% (v/v) FBS and 1X penicillin/streptomycin. Cells were incubated at 37 °C in a humidified 5% (v/v) CO₂ atmosphere.
3.3.5 Coating tissue culture plates with perlecan

Tissue culture plates were coated with the following proteins: full-length perlecan, H/C digested perlecan, PLN IV-1, IV-2, IV-3 or R3452Q. Uncoated, BSA, and H/C only served as controls. Tissue culture plates, 96-well and 24 well (Corning), were coated with 1 and 5 µg of protein diluted into 100 µl and 250 µl of PBS, pH 7.4 (Thermo Fisher Scientific), respectively. Plates were incubated overnight at 37˚C to allow the protein to adsorb to the plate surface. For experiments that required preheating, undiluted protein solutions were heated to 60˚C for 10 mins prior to being diluted in PBS. After allowing one day to adsorb to tissue culture plates, the remaining solution was removed, wells were rinsed with PBS, and cells were seeded. Cells were seeded at densities of 7,500 cells per well (96-well) or 250,000 per well (24-well) for analysis.

3.3.6 Assessing the bioactivity of perlecan fragments

After coating plates, ATDC5s and MSCs were seeded onto plates coated with various perlecan substrates. Cells were monitored over the course of 24 hrs. Images were captured at various time points with a Nikon inverted microscope in bright field mode with a 4 or 10X objective.

3.3.7 Cell attachment assay

Cell adhesion assays were performed in 96 well plates in serum free conditions. Cells were seeded at a density of 15,000 cells/well and allowed to adhere to various substrates for 1 hr. After 1 hr, wells were rinsed with PBS and cells were fixed with ice-cold 100% (v/v) methanol at -20˚C. After fixation, wells were rinsed with PBS and a single bright field image was taken at the center of the well using a taken at 4X objective. The total cells in each image were counted using the ImageJ particle count function software. Here
the software considers one cell equal to one particle (NIH, Bethesda, MD). The results were normalized such that the average number of cells bound to uncoated control plates equaled 100% attachment. Data is represented as a percentage of the total number of cells that attached to control plates.

3.3.8 ATDC5 cell dispersion and cell cluster size

Quantification of ATDC5 cell spreading was performed as previously (Grindel et al., 2014). As discussed previously, 96-well plates were coated with 1 µg of protein/well. Cells were seeded at a density of 7,500 cells per well in 5% (v/v) serum. After 24 hr the wells were imaged at 4X objective in bright field mode. Using ImageJ software, batch images were quantified for cell count, cell size, and area fraction. Cell dispersion values are equal to (cell count)/(cell size)*(area fraction). A higher value is more dispersed and a lower value is more clustered. To compare dispersion values, the cells have to be of the same type, seeded at the same density, and subconfluent when imaged.

3.3.9 Western blot analysis

ATDC5 cells were seeded onto 24 well plates, on plates either uncoated or coated with PLN IV-3 (5µg) for up to 24 hr. Cell lysates were collected with radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 1.0% (v/v) Triton-X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 1mM ethylenediaminetetraacetic acid (EDTA), 1mM ethylene glycol tetraacetic acid (EGTA), 50 mM Tris, pH 8.0, protease inhibitor cocktail mix at 1:100 dilution and phosphatase inhibitor cocktail mix at 1:100 dilution. Approximately 10% of the total protein extract was denatured at 99 °C with reducing sample buffer (final 60 mM Tris-HCl, 1% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 2% (v/v)
2-mercaptoethanol, pH 6.8 with tracking dye) for 5–10 min. Protein extract was
separated by SDS–polyacrylamide gel electrophoresis (PAGE) using a 5% (w/v)
Laemmli stacking gel and a 10% (v/v) Porzio and Pearson resolving gel at 150 constant
volts for approximately 80 min. Broad Range Marker (BioRad; Hercules, CA) was used
to determine apparent molecular weights. Separated proteins were transferred to 0.45 μm
pore nitrocellulose (Bio-Rad) in a tris-glycine buffer at 40 volts for 5 hr at 4°C.
Membranes were blocked in 3% (w/v) BSA Tris-buffered saline with 0.05% Tween-20
(TBST) for 2 hrs at RT. Antibodies were added to the block solution overnight on a 4 °C
shaker. GADPH was used at concentration of 1:5,000 and all other antibodies were used
at 1:1,000. Following three by 5 min TBST washes, the membranes were incubated
either with 1:50,000 goat anti-rabbit or sheep anti-mouse HRP conjugated antibody
(Jackson) in 3% BSA TBST for 1 hr at room temperature. Blots were rinsed three times
for 5 min each and signal intensities were detected using the ECL system (Pierce) as
described by the manufacturer. Blots were exposed to X-ray film, and signal intensities
were quantitated using Image J software. Pixel optical densitometric values were
obtained for each protein band signal. Intensity values were normalized to loading
control GAPDH or total FAK, SRK, or ERK protein. Data are expressed relative to
results from the uncoated control samples.
3.3.10 Cell proliferation assay
ATDC5 cells were seeded on 96-well plates uncoated or coated with PLN IV-3 for assay
of proliferative activity with the use of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-
carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] CellTiter 96® AQueous One
Solution Cell Proliferation Assay (Promega; Madison, WI). Cell metabolic activity was
quantified by measuring the conversion of MTS to formazan according to the manufacturer's instructions. At various time points over the course of two days in culture, MTS solution was added to each well for 1 hours, and light absorbance at 490 nm was detected by Tecan M1000 plate reader.

3.3.11 Alcian blue stain

ATDC5 cells were seeded into 24-well plates on normal or PLN IV-3 coated plates. Cultures were maintained for 14 days with media replaced. ECM production, a measurement of chondrogenic activity, was evaluated by staining cells with Alcian blue, a stain for sulfated and carboxylated acid mucopolysaccharides, and sulfated and carboxylated glycoproteins. Alcian blue staining was performed following established protocols (Newton et al., 2012). Cells were fixed in 4% (w/v) paraformaldehyde at room temperature for 5-10 minutes and stained with Alcian blue solution 1% (w/v) in 3% acetic acid (v/v), pH 2.5 M (Sigma) overnight. The following day, cells were washed in dH2O. Alcian blue-stained cultures were extracted with 6 M guanidine-HCl for 6 hr at room temperature and the OD was determined at 630 nm by spectrophotometry. Images were captured in bright field mode with a 4 and 10X objective.

3.3.12 RNA isolation and quantitative PCR

RNA was isolated 24 and 48 hr after seeding cells on appropriate substrate. RNA was extracted using 1 ml TRIzol reagent (Thermo Fisher Scientific) and suspended in 50 µl nuclease-free water. DNA-free kit (Thermo Fisher Scientific) was used to degrade remaining genomic DNA in accordance with manufacturers' directions. One microgram of RNA was reverse-transcribed using cDNA Supermix (Quantas, Gaithersburg, MD) as per the manufacturer's protocol and amplified using a CFX96 Real Time System.
For quantitative PCR (QPCR), B-R SYBR Green Supermix for IQ (Quanta Biosciences) was used for the reactions. The thermal cycling program was as follows: 95°C for 3 min, then 40 cycles of the following two steps: 95°C for 30s and 60°C for 15 s. All samples were analyzed as technical triplicates. Primer sets used for qRT-PCR are listed in Table 3.1 below. Reactions were normalized to ppiα as suggested by Zhai et al. (2013) and relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method.

### Table 3.1 Oligonucleotide primers used for QPCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>acan (F)</td>
<td>CAGGGTTCCCAGTGTTCACTG</td>
</tr>
<tr>
<td>(R)</td>
<td>CCAGAAGACTCTCCACTG</td>
</tr>
<tr>
<td>atf3 (F)</td>
<td>CGAAGACTGGAGCAAATGAT</td>
</tr>
<tr>
<td>(R)</td>
<td>CAGGTTAGCAAAAATCCTCAAAC</td>
</tr>
<tr>
<td>cdh2 (F)</td>
<td>AGCGCAGTCTACCGAAGG</td>
</tr>
<tr>
<td>(R)</td>
<td>TCGCTGCTTTTCATACTGAACCTTT</td>
</tr>
<tr>
<td>cdkn1c (F)</td>
<td>GTCCAGGAGAAGGAGCAAG</td>
</tr>
<tr>
<td>(R)</td>
<td>GAGAGTCGTTCGATTG</td>
</tr>
<tr>
<td>col2a1 (F)</td>
<td>CTCATCCAGGGCTCCATGA</td>
</tr>
<tr>
<td>(R)</td>
<td>TCCCTACAGGGCAGGTATGT</td>
</tr>
<tr>
<td>ppiα (F)</td>
<td>CCGTCTCCTCGAGGTGT</td>
</tr>
<tr>
<td>(R)</td>
<td>TGTAAGTCACACCCTGGCACAT</td>
</tr>
<tr>
<td>sox9 (F)</td>
<td>GAGGCCACGAAGACAGTCA</td>
</tr>
<tr>
<td>(R)</td>
<td>CAGCGCTTGAGATAGCATT</td>
</tr>
</tbody>
</table>

### 3.3.13 Statistics

For cell the cluster and dispersion assay, MTS assay, Alcian blue staining, QPCR and western blot analyses an unpaired student’s T-test was utilized to determine statistical significances. All experiments were performed in triplicate wells and repeated at least three times, and the means and standard deviations were plotted using the statistical software GraphPad Prism 5.
3.4.0 Results

3.4.1 PLN IV-3 promotes cell-cell adhesion

Perlecan activity was determined by changes in cell morphology. Figure 3.3 A shows schematic diagram illustrating the location along the core protein from which the subdomains were created. Below the diagram are images captured 1hr after seeding (figure 3.3 B-E). Figure 3.3 B is a representative image of ATDC5 cells in a monolayer culture on normal uncoated tissue culture plates. BSA-, IV-1-, IV-2-, and full-length perlecan core only (H/C digested) -coated plates did not produce a response and appeared similar to figure. 3.3 B. Full-length perlecan contain GAG attachments was used as control, considering its known role in promoting chondrogenesis (figure 3.3 C). As expected, full-length perlecan with GAGs prevented early cell attachment and stimulated cell rounding. Subdomain IV-3 had a more pronounced effect, resulting in large cell clusters resembling in vitro precartilage mesenchyme condensation models (figure 3.3 D). Although R3452Q produced a response, the effect was not as strong as IV-3 (figure 3.2 E).
Figure 3.3 Purified recombinant PLN IV-3 substrate promotes cell-cell adhesion. (A) A schematic representation of the domain structure of perlecan and purified recombinant subdomains. The R3452Q mutation is located within the 19th Ig module of domain IV, as indicated by the arrow. Below the perlecan schematic are representative images of ATDC5 cells after 1 hr of plating on various perlecan substrates. (B) ATDC5 cell spreading occurred on the control uncoated plates. The following substrates produced a similar response to control uncoated, H/C digested intact perlecan, H/C enzyme control mix, BSA, and subdomains IV-1 and IV-2. (C) Full-length perlecan with intact GAG side chains, (D) domain IV-3, and (E) R3452Q prevented cell attachment and spreading, and promoted cell-cell adhesion. Cell-clustering was most noticeable in cells grown on IV-3. Representative bright field images were captured using a 10X objective. Magnification of insets are shown in bottom right corner of each image. Scale bar indicates 200 μm.
3.4.2 Far-UV CD spectral analysis of IV-3 and R3452Q

The secondary structural characteristics of IV-3 and R3452Q were compared by far-UV circular dichroism (CD). CD spectra are plotted as wavelength versus mean residue ellipticity values. CD spectra revealed both proteins have a typically beta-sandwich-like confirmation with a maximum at 202 nm and minimum at 217 nm when kept at ambient temperatures (figure 3.4 A-B). Structural prediction software (DichroWeb) indicated the two proteins are composed of approximately 50% beta strands. Protein stability was assessed by gradually increasing the sample temperature from 20 to 90\(^{\circ}\)C, with spectral measurements recorded at every 10\(^{\circ}\)C. Both proteins began to unfolding at 60\(^{\circ}\)C, indicated by shifts in the maximum and minimum wavelength values. The subdomains appeared to experience an intermediate conformation at 60\(^{\circ}\)C. This conformation only existed within a specified temperature range, given the molecules were completely denatured once temperatures reached 70\(^{\circ}\)C.
Figure 3.4. Far UV CD structural analysis of PLN IV-3 and SJS mutant R3452Q. Perlecan domain IV-3 (A) and R3452Q (B) spectra were analyzed over a temperature range of 20 to 90˚C, with measurements recorded every 10 °C. Perlecan domain IV-3 (A) and R3452Q (B) displayed similar changes in their spectra with increasing temperature. Both proteins partially unfolded at 60˚C and completely lost their secondary structures at 70˚C. Each data point represents an average of three separate experiments.
3.4.3 Thermal unfolding of PLN IV-3 and R3452Q

To further understand the stability of PLN IV-3 and R3452Q, thermal unfolding was monitored at 202 nm, corresponding to the maximum peak in figure 3.4. CD ellipticity values are normalized to fraction folded, ranging from 1 to 0, relative to the low temperature value. Curves revealed melting temperatures of 63.72 °C and 62.39 °C for PLN IV-3 and R3452Q respectively (figure 3.5). Based on CD analysis there appears to be no major structural difference between normal IV-3 and R3452Q.
Figure 3.5 Thermal denaturation of PLN IV-3 and R3452Q monitored by CD spectroscopy. Protein stability was monitored by absorbance at 202 nm. The data are normalized to show the folded fraction as a function of temperature for PLN IV-3 (black points) and R3452Q (unfilled). Melting temperatures ($T_m$) were determined by fitting the curves to a Boltzmann sigmoidal equations (IV-3, \( T_m = 63.72 \, ^\circ\text{C} \); R3452Q, \( T_m = 62.39 \, ^\circ\text{C} \)). Fitted curves are shown as black (IV-3) and dashed (R3452Q) lines. Error bars in represent SEM.
3.4.4 PLN IV-3 aggregation

For CD analysis, a relatively low protein concentration had to be used (~ 2.67 μM). This indicated that our sample was likely aggregating, at least when measured at ambient temperature. To determine the extent of PLN IV-3 aggregation, AFM images were taken of PLN IV-3 adsorbed onto a mica substrate. Even at a concentration of 133 nM, which is much lower than the sample concentration for CD measurements, aggregation was apparent. Representative AFM images are shown in figure 3. X. On the left is an image captured of freshly thawed PLN IV-3 that was equilibrated to 37 °C for 5 mins prior to coating. The right-hand figure represents results after the protein sample was heated to 60 °C for 10 mins before coating and imaging. We show here that by heating perlecan we could break up large aggregates into what appeared to be single monomers. PLN IV-3 aggregation was also detected in size-exclusion chromatography. A chromatogram of PLN IV-3 fractionated into two forms is provided in the appendix.
Figure 3.6 PLN IV-3 aggregates visualized by AFM. PLN IV-3 at 10 µg/ml in PBS was coated onto mica substrates and imaged in ScanAsyst mode in air. Height maps were generated to compare aggregation between (A) freshly thawed and (B) heated (37°C for 30 min) PLN IV-3 samples. The large protein complexes observed in A completely broken up into monomer size components when heated, shown in B. Note the differences in scan size between the two images, as shown below the maps. Additionally large increases in height were observed, as indicated by scales on right side of the maps.
3.4.5 R3452Q SJS mutation inhibits cluster formation

As a measure of protein activity, ATDC5 cell dispersion (spreading) vs clustering was quantified. For this assay, cells were grown on tissue culture plates (7,500/0.32 cm²-96-well) coated with appropriate protein (1µg) for 24 hr. At which point cells were imaged for dispersion analysis. Representative images for clustered and dispersed cells are shown on the left and right sides of the graph in figure 3.7 A, respectively. Data is normalized to percent dispersion relative to BSA-coated plates (top bar of figure 3.7 A). Data are represented as dispersion value relative to the control BSA-coated plates. IV-3 induced cell clustering, represented by low dispersion value of 35%. The R3452Q mutation reversed this effect, although, there was still a 20% reduction in cell spreading compared to the BSA control.

To test the activity of the intermediate conformation, as observed by CD, proteins were denatured by heating protein solutions to 60⁰C for 10 mins prior to coating tissue culture plates. Interestingly, denatured protein substrates (IV-3 60⁰C and R3452Q 60⁰C) induced a greater clustering effect than their native counterparts. The IV-3 60⁰C substrate decreased cell spreading down to 17%. Similarly, the unfolded R3452Q reduced dispersion down to 22%.

These findings were also reflected in cell cluster size (figure 3.7 B). A low cell dispersion index tended to correlate with increased cell cluster size. The exception being R3452Q, which failed to increase cell cluster size. The largest cell aggregates were observed in those cultured on denatured substrates.
Figure 3.7 ATDC5 cell clustering affected by altered perlecan domain IV-3 substrates. ATDC5 cell clustering occurred on all substrates (excluding BSA control) but was greatly reduced when introduced to a R342Q mutant substrate. Thermal unfolding of IV-3 and R3452Q (60°C for 10 minutes prior to coating) significantly enhanced cluster activity. For quantification, images were taken at 4X objective 24 hr after seeding. (A) Cell dispersion and (B) Cluster size were quantified using ImageJ software. In A values are reported as a percentage of the total cell spreading of cells grown on BSA-coated plates. Representative clustered (left) and dispersed (right) processed images are shown in A. In B values are reported as a ratio relative to BSA. An unpaired Student’s t-test was used to determine statistical significances; *p value < 0.05; **p value < 0.001; ***p-value <0.0001. Error bars represent SEM.
3.4.6 IV-3 induced nodule formation reassembling mesenchyme condensation

To enhance the ATDC5-PLN IV-3 response, we adapted our *in vitro* system to a large plate format with higher cell density. The conditions were 250,000 cells per well in a 24-well plate (1.9 cm²) coated with 5 µg of protein. In addition, to maximize perlecan activity we adopted the 60 ºC pretreatment as our standard for PLN-IV3 studies. These slight modifications allowed us to create large cartilage-like nodules. As a control, ATDC5 cells were grown on uncoated plates where they remained in a monolayer and evenly dispersed throughout the plate (figure 3.8 A). When cultured on PLN IV-3 ATDC5 cells immediately aggregated after seeding leading to the formation of tight dense clusters as seen in the top right of figure 3.8 B. Nodules were also seen forming in regions of the plate with high cell density, as indicated by the black arrow at the bottom of figure 3.8 B. These conditions were used for all protein and mRNA analysis.
Figure 3.8 ATDC5 cells, in a high density culture, aggregate to form clusters resembling cartilage nodules after 24 hr of being seeded on a purified PLN IV-3 substrate. ATDC5 cells were cultured on normal tissue culture plates (A) or PLN IV-3 coated substrate (B) for 24 hr. ATDC5 cells spread and grew in a monolayer on uncoated plates, while those grown on a PLN IV-3 substrate formed large tight clusters. Representative images were captured at 24 hr. Although not present the first day, clusters continue to develop later in regions of high cell density, as indicated by the arrow in B. Western blot analysis was used to show PLN IV-3 was not being degraded. Scale bar represents 200 μm.
3.4.7 IV-3 interferes with cell-substrate attachment by suppressing FAK/SRC activity. Cell spreading is regulated by focal adhesion dynamics. IV-3 induced clustering begins with the disruption of cell-substrate attachment, as demonstrated in a cell adhesion assay shown in figure 3.7 A. 7,500 cells were seeded on uncoated or IV-3 coated plates (96-well) and allowed to attach for 1 hr. Unattached cells were removed and the number of cells attached was counted. Results are normalized to the percentage of adhesion of the uncoated, which was assumed to be 100%. IV-3 clearly inhibited cell attachment with only 7% relative to the control (figure 3.9 A).

Given FAK and SRC are involved in cell adhesion, I wanted to know whether or not the activity of these two proteins was influenced by PLN IV-3. Western blots were performed using phosphospecific antibodies targeted to active FAK (Try 397) or SRC (Try 416). Extracts for analysis were collected from ATDC5 cells at 24 hr. Shown in figure 3.9 B, phospho-FAK (pFAK) levels were greatly reduced in cells grown on IV-3. A similar suppression is seen with phospho-SRC (pSRC), shown in figure 3.8 C. These results demonstrate perlecan’s ability to interfere with focal adhesion.
Figure 3.9 PLN IV-3 inhibits cell-substrate attachment and suppresses FAK/SRC activation. (A) ATDC5 cells were assayed for adhesion to subdomain IV-3. After 1hr, only 7% of cells attached to IV-3. Loss of cell-adhesion correlated with reduced FAK (B) and SRC (C) activity, as determined by western blot analysis. Cell lysates were collected at 24 hr. Graph B shows results from densitometry analysis with pFAK antibody normalized by total FAK. Graph C shows results from densitometry analysis with pSRC antibody normalized by total SRC. Representative western blots are shown below each graph. An unpaired Student’s t-test was used to determine statistical significances; *p-value <0.05; **p-value <0.01. Error bars represent SEM.
3.4.8 PLN IV-3 suppression of FAK/SRC activation negatively impacts ERK signaling

We investigated ERK signaling because of its role in the regulation of the adhesion/cytoskeletal network. We found dephosphorylation of FAK and SRC coincides with reduced phosphorylation of ERK, a major signaling component of the MAPK cascade. This was demonstrated by western blot analysis comparing the intensity of phospho-MAPK (pERK1/2; Thr202/Tyr204) antibody detection to that of total p44/42 MAPK (ERK1/2). Western blot produces two bands, corresponding to molecular weights of 44 kDa and 42 kDa for ERK1 and ERK2 respectively. Results demonstrated PLNIV-3 suppressed pERK1/2 by over 50% (figure 3’10).
Figure 3'10 PLN IV-3 suppresses ERK1/2 activation. A western blot were performed with ERK and pERK antibodies in ATDC5 cells grown on uncoated and IV-3 coated plates. The graph below shows results from densitometry analysis with pERK antibody normalized by total ERK. Cell lysate was collected at 24 hr. A statistical difference was determined by an unpaired Student’s t-test was used determine statistical significances; ***p-value <0.005. Error bars represent SEM.
3.4.9 Downstream effects of ERK suppression mediated by PLN IV-3

To further investigate perlecan-mediated FAK/ERK signaling, downstream events associated with kinase inhibition were examined. As a substrate, PLN IV-3 reduced ATDC5 production of total FoxM1 protein by almost 50% (figure 3’11 A). FoxM1 is well-known controller of cell cycle progression, although this is the first report of its involvement in chondrogenesis. As expected, loss of FoxM1 reduced cell proliferation, measured by MTS assay for metabolic activity (figure 3’11 B).
Figure 3’11 IV-3 reduced Foxm1 protein levels and decreased cell proliferation. (A) FoxM1 expression was compared in ATDC5 cells seeding on either uncoated tissue culture plates or on a PLN IV-3 substrate. A representative western blot performed with FoxM1 and GAPDH. The graph shows results from densitometry analysis with FoxM1 antibody normalized to GAPDH. (B) Proliferation rate comparison of ATDC5 cells grown on standard tissue culture plates or on a PLN IV-3 coated substrate using MTS assay over 48 hr. A statistical difference was determined by an unpaired Student’s t-test was used determine statistical significances; **p-value <0.01; ***p-value <0.0001. Error bars represent SEM.
3.4.10 PLN-IV3 repressed transcriptional output of FoxM1

Here, the expression of FoxM1 controlled genes involved in cell-cycle progression were investigated. Quantitative PCR was performed to analyze cell cycle inhibitors Cyclin-dependent kinase inhibitor 1C (cdkn1c) and activating transcription factor 3 (atf3) in response to PLN IV-3. Both cdkn1c and atf3 expression increased in PLN IV-3 grown cultures over the course of two days (figure 3’12 A & B)
Figure 3’12 IV-3 controls expression of Cdkn1c and atf3 mRNA. Cdkn1c and atf3 mRNA levels were determined 1 and 2 days after ATDC5 cells were seeded. Transcripts levels are normalized to ppai. Data is represented as a fold change relative to day 1 uncoated. (A) IV-3 increased Cdkn1c expression 2-fold at days 1 and 2. (B) Atf3 expression reached almost 2-fold higher after day 2. The bars represent the means ± SEM of triplicate determination of independent samples in each case. A statistical difference was determined by an unpaired Student’s t-test was used determine statistical significances; *p-value <0.05; **p-value <0.01; *** p-value <0.001.
3.4.11 PLN IV-3 delays proteoglycan synthesis

GAG synthesis, an indication of chondrocyte differentiation, was measured by Alcian blue staining. ATDC5 cultures were examined at days 1, 2, 4, 7, 10 and 14 post-seeding (figure 3.13). GAG production in PLN IV-3 grown cultures was lower during the first week of culture, but by day 10 GAG secretion was closer to uncoated plates. These results agree with the MTS assay and suggest IV-3 grown cells are not as metabolically active. In both culture conditions matrix production sharply increased from day 4 to 7. Soon after proteoglycan levels flattened out, with relatively modest changes throughout the last week of culture.
Figure 3'13 PLN IV-3 delays matrix production. ATDC5 cells were grown for 14 days on either uncoated or IV-3 coated plates. Alcian blue staining was performed to measure GAG production during this time period. An unpaired Student’s t-test was used determine statistical significances; *p value < 0.05; **p value < 0.005; ***p-value <0.0001. Error bars represent SEM where N = 3.
3.4.12 PLN IV-3 induced expression of genes associated with mesenchyme condensation

The mRNA levels of precartilage condensation markers *sox9* and *cdh2* were examined in PLN IV-3 induced nodules by QPCR. Relative transcripts levels were measured at 1 and 2 days post ATDC5 seeding to capture early gene changes that occur during cell condensation. Transcripts levels for *sox9*, a key regulator of chondrogenesis, increased after 24 hr (figure 3’14 A). By day 2 *sox9* expression in PLN IV-3 cell clusters was closer to control cells, although it was still significantly different. A similar trend was observed for *cdh2*, whose gene product is important for cell-cell adhesions (figure 3’14B). The lower fold change observed at day 2 likely stems from increased proliferation of non-clustered cells surrounding the nodules, as see in figure 3.8.
Figure 3: PLN IV-3 controls expression of early cartilage-associated genes *sox9* and *cdh2*. mRNA levels were determined 1 and 2 days after ATDC5 were seeded. Transcripts are normalized to *ppai*. Data is represented as a fold change relative to the transcript levels of ATDC5 cells on uncoated plates. (A) PLN IV-3 increased *sox9* expression almost fold change within 24 hr of culture. By day 2 the difference was less but still significantly different from the control (B) A similar trend was observed for *cdh2* expression, with only a modest change in fold expression between PLN IV-3 and controls. The bars represent the means ± SEM of triplicate determination of independent samples in each case. A statistical difference was determined by an unpaired Student’s t-test was used to determine statistical significances; *p*-value <0.05; **p*-value <0.001; ***p*-value <0.001.
3.4.13 PLN IV-3 induces gene expression of early chondrogenic markers

The mRNA levels of early cartilage markers *col2a1* and *acan* were examined in PLN IV-3 induced nodules by QPCR. In contrast to the Alcian blue staining, both *col2a1* and *acan* transcript levels increased in PLN IV-3 cell clusters (figure 3’15).
Figure 3'15 PLN IV-3 increases transcription of cartilage matrix genes *col2a1*, and *acan*. mRNA levels were determined 1 and 2 days after ATDC5 were seeded. Transcripts are normalized to *ppai*. Data is represented as a fold change relative to the transcript levels of ATDC5 cells on uncoated plates. (A) PLN IV-3 increased *col2a1* expression of the course of two days. Highest fold change was observed at day 1 where PLN-IV3 induced *col2a1* expression by 2 fold. (B) A similar trend was observed for *acan* expression with over a 2-fold induction seen at day 1. The bars represent the means ± SEM of triplicate determination of independent samples in each case. A statistical difference was determined by an unpaired Student’s t-test was used determine statistical significances; *p*-value <0.05; **p*-value <0.001; *** p*-value <0.001.
3.4.14 PLN IV-3 suppressed FAK activation in bone marrow-derived MSCs

Bone marrow-derived MSCs have the capacity to differentiate in vitro to various mesenchymal tissues including bone and cartilage. MSCs were examined to see whether or not they responded to PLN IV-3. Figure 3’16 A-B are images obtained 24 hr after seeding MSCs on either uncoated or PLN IV-3 coated plates. On an uncoated surface cells dispersed evenly throughout the plate (figure 3’9 A). Whereas, those grown on IV-3 formed a reticular network with void spaces (figure 3’9 B). FAK, SRC, ERK activity was assessed by western blot analysis (figure 3’16 C). Results are graphed in figures 3.9 D-F. Unlike ATDC5 cells, only FAC activity was suppressed (figure 3’16 D). SRC and ERK signaling was not affected (figures 3’16 E and F).
**Figure 3’16 Bone marrow-derived MSCs cultured on IV-3.** PLN IV-3 activity was assessed in bone marrow-derived MSCs. Figures A and B are representative bright field images (4X objective) of cells cultured on either uncoated or IV-3 coated plates for 24 hr. Scale bars indicate 200 µM. (C) FAK, SRC or ERK activity was assayed by western blot. Western blot analyses are shown in the bottom graphs D-F. As shown in D, IV-3 suppressed FAK activation, but failed to affect SRC and ERK despite noticeable changes in cell phenotype. The bars represent the means ± SEM of triplicate determination of independent samples in each case. A statistical difference was determined by an unpaired Student’s t-test was used determine statistical significances; **p p-value <0.001.
3.5.0 Discussion

3.5.1 Results Summary

The purpose of this study was to identify perlecan-derived signaling elements that influence chondrogenesis. The function of a perlecan subdomain, PLN IV-3, was characterized in a chondrogenic cell line, ATDC5. When present as a cell substrate, PLN IV-3 interfered with cell-substratum attachment and induced cells to form cell-cell interactions. The anti-adhesiveness of PLN IV-3 was mediated through FAK/SRC activity. It is likely there are multiple signaling pathways are affected by this response, considering the utility of FAK. One of which is the MAPK/ERK pathway, shown here to be less active in cells responding to PLN IV-3. This is turn affects the downstream target FoxM1 and increases transcription of cell cycle inhibitory genes \( atf3 \) and \( cdkn1c \). In all, I have identified a region within the perlecan core protein that has implications in early stage condensation, possibly controlling cell-matrix interactions and cell proliferation.

3.5.2 FAK activity in mesenchyme condensation

The studies presented here demonstrate perlecan can interfere with formation of focal adhesions by suppressing FAK and SRC phosphorylation/activity. This had direct relevance to endochondral bone formation, considering cell-matrix interactions drive mesenchyme cell condensation and the subsequent mesenchymal cell differentiation to a chondrogenic lineage (Bang et al., 2000). Suppression of FAK, along with SRC, has been shown to induce chondrogenesis (Pala et al., 2008). The idea being the loss of cell-ECM interactions prevent cell migration and drives cell-cell interactions leading to condensation. Along the same lines, blocking cell-matrix adhesion, via FAK inhibition, prevents chondrocyte death following mechanical injury (Jang et al., 2014)). Inhibition
of FAK, using the pharmacological compound PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) PP2, has been shown to increase express levels of collagen type 2, aggregan, and Sox9 in dedifferentiated chondrocytes (Shin et al., 2015). Similarly, PP2 was used to induce chondrogenesis in ATDC5 cells, demonstrated by increased expression of early markers Sox9, collagen 2, aggregan, and xylosyltransferases (Bursell et al., 2007). Data presented in this study clearly points to FAK/SRC involvement in perlecan domain IV-3 induced clustering. The PLN IV-3 induced phenotype resembles cell aggregates formed in in vitro mesenchymal condensation models. From cartilage-engineering standpoint, identifying a bioactive molecule that induces cell-cell interactions is a laudable discovery, particularly at time where the current field is looking to develop cell condensation-inducible scaffolds. It’s becoming increasingly clear that the best tissue-engineering strategies will be ones implementing cues from nature and using molecules with physiological relevance. Research points to perlecan as an attractive candidate for such purposes. In theory, perlecan and other biological ECM components could be used to develop scaffolds that mimic the native tissue environment. The goal being to use native-like scaffolds, along with specific stimulatory signals, to provide a more in vivo like cellular response, one that many current artificial materials fail to produce to the extent needed to successfully engineer cartilage.

3.5.3 ERK MAPK signaling in cartilage development

Once this perlecan-induced phenotype was identified, the concern became elucidating the signaling mechanism by which this cellular response was achieved. There is growing body of work supporting the cooperative roles of FAK and MAPK in chondrocyte
biology. ERK is a major component of the MAPK cascade and is critical for skeletal development and implicated in a number of human skeletal syndromes. In vivo studies have shown ERK1 and ERK2 regulate chondrocyte terminal differentiation during endochondral bone formation (Chen et al., 2015).

Fibronectin is a known activator of human articular chondrocytes through FAK and ERK (Gemba et al., 2002). Our results indicate an opposing role of perlecan IV-3, which inactivates ERK signaling. This is often the case with ECM molecules and is a tactic by which the ECM has dynamic control of cellular processes. MAPK signaling is not only important for developing cartilage tissues, but also has tremendous influence in both mature healthy and damaged tissues. Tissue mechanical forces themselves can activate MAPKs and promote inflammatory responses, such occurs at the intervertebral discs of the spine and in articular cartilage lining joints (Pratsinis et al., 2015). In early-stage osteoarthritis the FAK/MAPK pathway plays a role in protecting cartilage from damage (Xia et al., 2015). Interestingly perlecan expression is elevated in osteoarthritis patients (Tesche and Miosge, 2004). It is proposed this response is carried out by the chondrocytes to preserve their matrix environment.

The ERK pathway is a well-known regulator of many transcription factors important for cell proliferation. PLN IV-3 inhibition of ERK1/2 therefore suggests perlecan is somehow linked to cell cycle progression. To further explore PLN IV-3/ERK signaling I chose to investigate the transcription factor FoxM1, a molecule that is a well-known ERK target, but whose function has yet to be studied in the context of cartilage biology.

FoxM1 Null mice results in a lethal phenotype caused by defects in mitosis (Kalin et al., 2011). FoxM1 is ubiquitously expressed in proliferation cells with expression peaking at
the G2/M phase of the cell cycle (Ma et al., 2005). FoxM1 is found in the cytoplasm
during late-G1 and S phases, but just before entry into the G2/M phase becomes
phosphorylated by the Raf/MEK/MAPK pathway and is translocated to the nucleus (Ma
et al., 2005). FoxM1 contains two ERK1/2 phosphorylation target sequences, although
direct activation of FoxM1 by ERK has not been shown (Ma et al., 2005). Transactivity
of FoxM1 is enhanced by active MEK1. Interestingly, FOXM1 activates its own
expression in a positive autoregulatory loop manner (Halasi and Gartel, 2009). Kalin,
Ustiyan, and Kalinichenko (2011) found, by immunostaining, that FoxM1 is expressed in
cartilage during murine embryogenesis. As of date, no one has reported experimental
data investigating FoxM1 in chondrogenic cell line. We show that the widely used
prechondrocyte cell model ATDC5 express FOXM1 protein. Additionally, when the
ATDC5 cells were grown on PLN IV-3 coated substrates for 24 hr foxm1 protein levels
decreased, coinciding with reduced phosphorylation of ERK1/2 and loss of cell
dispersion. This strongly agrees with the literature in that ERK FoxM1 signaling is
associated with an invasive phenotype (Xie et al., 2015)).

FoxM1 has emerged as a fundamental regulator of mitotic division and cell proliferation.
FoxM1 controls cell cycling progression by regulating G1/S and G2/M phase transition
genes. In developing pituitary gland, FoxM1 is present in actively proliferating cells,
whereas in the cells with little to expression high levels of the cell cycle inhibitor
p57/cdkn1c were found (Bilodeau et al., 2009; Calderon et al., 2015). Remarkably,
reduced FAK activation and loss of cell-ECM adhesion increased transcriptional output
of cdkn1c, along with atf3, a member of the CREB protein family of transcription factors
and a also cell cycle inhibitor (Bursell et al., 2007). ATF3 mRNA levels increase during
chondrocyte differentiation, which appears to facilitate cell cycle exit and lead to terminal differentiation of chondrocytes (James et al., 2006). As expected, increased transcript levels of *cdkn1c* and *atf3* lead to decreased cell proliferation as measured by MTS assay.

3.5.4 Conclusion

In conclusion, this work identified a novel active element within the perlecan core protein with chondrogenic potential. The subdomain IV-3 stimulated cell-cell adhesion by interfering with cell-ECM attachment. IV-3 induced morphological changes were accompanied by deactivation of MAPK signaling, a conventional pathway implicated in cartilage and bone formation. In conclusion we used IV-3 to control cell dispersion and proliferation, two key cellular processes involved in mesenchyme condensation.
4.0 Chapter 4 Summary, General Discussion and Future Directions

The purpose of this dissertation is to increase our understanding of perlecan’s role in skeletal tissues. These studies investigate two aspects of perlecan: 1) the physical properties of perlecan important to bone mechanotransduction and 2) regulatory signaling mechanisms of the perlecan core protein involved in chondrogenesis. While at first glance unrelated, these two aims share a common theme in that they both investigate perlecan’s role in controlling bone mineralization.
4.1.0 Summary: Single molecule force measurement of perlecan

The skeleton is affected by a large number of genetic diseases and developmental disorders. Often these conditions result in loss of bone tissue and decreased bone quality, as occurs in osteoporosis, which is one of the most common skeletal diseases. Individuals with osteoporosis are tremendously high at risk for developing bone fractures, most commonly in the bones of the hip, spin, and wrist. Although there are several risk factors that often influence the development of osteoporosis, anyone can be affected. Current treatment options focus on healing and preventing fractures. A well designed comprehensive treatment plan can help minimize the effects of the disease, although treatment options can be very expensive and individuals still have a diminished quality of life. Bone mechanotransduction plays a significant role in maintaining bone health. Understanding how bone adapts and remodels its architecture is crucial for combating osteoporosis and other diseases affecting bone tissue. It’s well known the mechanical environment is an important regulator of bone homeostasis. Osteocytes are the primary sensors of the mechanical environment that drive bone adaptation. The mechanism by which osteocytes sense their environment is currently a major area of focus. It was recently discovered that the perlecan-rich osteocyte pericellular matrix regulates solute transport and mechanosensing within the lacunar-canalicular system. The biomechanical properties of perlecan are central for its role within the osteocyte pericellular matrix. These properties were investigated by AFM. The major findings of this study are summarized below.

1. The size and structural features of perlecan were characterized using high resolution AFM imaging techniques. Images revealed the perlecan exhibited a
linear-shape confirmation that was composed of a series of globular modules with
diameters of 2-4 nm and heights of 5-10 nm. The core itself appeared flexible
taking on a variety of profiles with a measured persistence length of 19 ± 5 nm.
The average contour length of the perlecan core protein measured from 170 ± 20
nm. This demonstrated perlecan was of sufficient size to transverse the annular
gap of the lacunar-canalicular system, which is approximately 100 nm, and tether
the osteocyte cell body to the bone tissue matrix.

2. In order to function as function osteocyte tether, perlecan needs to withstand
substantial amount of drag forces created by interstitial fluid flow. Perlecan
withstood stretching forces over 100 pN, which is well above the estimated drag
forces within the LCS. Under tensile stress perlecan stretched beyond its
measured end-to-end length without compromising its structural integrity.

3. Full-length intact perlecan is relatively flexible with a persistence length of 1.2
nm and an elastic constant of 2000 pN. Although these measurements account for
the GAG side chains as well.

4. Upon removal of the GAG side chains the persistence length increased to 22 nm,
consistent with predictions based on AFM images. The protein core has a mean
elastic constant of 890 pN and corresponding Young's modulus of 71MPa.

5. Under normal circumstances, perlecan exists as a stable intact protein, and only
when chemically denatured by oxidizing the disulfides did perlecan unfold on
response to tensile mechanical stress.

The conclusion of this project is that perlecan has the physical properties that would
allow it to act as a strong, but elastic, tether in the LCS.
4.1.1 Future directions

This study provides supporting evidence that perlecan functions as an osteocyte tether. Subsequent studies need to examine the mechanism by which PCM perlecan forms functional tethers. A model is proposed in figure 4.1. To function as a mechanosensitive tether, perlecan needs to form strong stable interactions at the osteocyte cell surface and bone matrix wall. Based on previous data, we propose perlecan is anchored to the osteocyte cell surface through its C-terminal end, domains IV and V. Domain IV is capable of forming interactions with other matrix components that express Ig modules such as the cell adhesion molecules (CAMs). The binding between CAMs and heterotypic Ig modules is typically of low affinity (<100-200nM), but can be stabilized in a “zipper-like” manner by the multi-valency of tandem Ig repeats (Knox et al., 2001; Koper et al., 2012; van der Merwe and Barclay, 1994). We have preliminary data showing perlecan domain IV can binds to the ectodomain of various CAMs (See appendix). Domain V interacts with cell surface integrins, with a measured affinity to β1 integrin of 80-200 nM (Bix et al., 2004; Lee et al., 2011; Nyström et al., 2009).

Intriguingly, conditional knockout of β1 integrins in cortical osteocytes was found to prevent disuse bone loss (Phillips et al., 2008), similarly to our observation in perlecan Hypo. On the other end, domain I is likely to interact with the mineralized bone matrix wall. Domain I, via GAG side chains, can bind heparin-binding domains, which are found in bone matrix proteins collagen type1, fibronectin and osteopontin. Interestingly, a deficiency of these ligands such as osteopontin rendered mice resistant to unloading induced bone loss (Ishijima et al., 2002), similar to our observation in Hypo mice.
Our lab in collaboration with Dr. Liyun Wang’s research group at the University of Delaware already has developed a research strategy to determine the mechanism by which perlecan forms an osteocyte tethers. The studies we have proposed will use latest single-molecule techniques to identify perlecan anchorage molecules. Our plan is to have perlecan domains, attached to the AFM cantilever tip, brought into contact with the appropriate substrate, either integrins/CAMs of the osteocyte cell membrane or matrix proteins of the canalicular wall. This method will provide force data relevant for mechanosensing, such as the binding strength between perlecan and anchoring proteins.

During my time Farach-Carson lab, I already have developed the constructs necessary to carry out these experiments.

We first want to spatially map the interactions between perlecan and integrins (β1/β3) along the osteocyte cell body and cell process. For these studies, we have proposed to use the osteocyte cell line MLO-Y4 and harvested osteocytes from long bone of mice. Co-localization of endogenous perlecan protein with cell surface integrins will be determined by immunohistochemistry and confocal imaging. Our lab has various perlecan antibodies readily available to carry out these studies. Cell binding assays will be carried out using labeled exogenous recombinant perlecan fragments. We will use siRNA to silence endogenous perlecan expression and candidate binding targets. Second, we will investigate interactions of perlecan domain IV with osteocyte CAMs. In solid-phase binding assays, the PLN IV-3 interacted with NCAM2, NrCAM, and EpCAM. The expression of these CAMs will be tested in various osteocyte cell lines. Perlecan’s anchorage to the LCS matrix wall will be determined by investigating perlecan binding to various bone matrix component such as collagen 1, fibronectin and osteopontin.
Currently domain I is thought of as the part of perlecan that anchored to the LCS bone matrix wall. Similar to the proposed domain IV-V AFM studies, the interaction of domain I with and without its GAG side chains will be tested on the three bone matrix proteins. Binding specificity will be determined using blocking/competing antibodies to bone proteins. This study will then be performed on bone tissue slices. With this information we will be able identify perlecan anchors and determine their binding strengths.

The ultimate goal is to determine the roles of perlecan tethers in bone adaptation and osteocyte mechanosensing. Functional studies will be carried out in vivo using perlecan deficient mice. These studies will directly measure how the loss of perlecan within the PCM alters bone adaptation. Bone tissue structure and gene expression will be evaluated under loading and unloading conditions. Bone adaptation will be achieved by axial tibia loading and hind-limb suspension. Perlecan’s role in osteocyte mechanosensing will be assessed by studying how the perlecan deficient PCM regulates load-induced cellular stimulation and osteocyte calcium signaling. Novel tracer velocimetry approaches will be used to determine load-induced flow velocity, hydrodynamic shearing forces (cell membrane) and drag forces (perlecan tether).
Figure 4.1 **Perlecan as a mechanosensing osteocyte tether.** (A) AFM image of perlecan’s core protein with predicted domain orientation. Known bone matrix contacts are listed and the domain where interaction is formed. Note the flexible nature of the core protein (green arrow; domains IV-V). (B) A proposed perlecan sensing tether in LCS (not to scale) with its c-terminal (green arrow) anchored to the cell surface, via integrins or CAMs. Domain I, because of its HS chains, likely binds to the surface of the bone matrix wall. Image courtesy of Dr. Wang.
4.2.0 Summary: PLN IV-3 induces ATDC5 cell condensation

The condensation of mesenchymal stem cells is a prerequisite for subsequent cartilage and bone formation. Improving our understanding of this process will ultimately aid in the developing of therapeutics to combat skeletal diseases and disorders, such as SJS. In this study we sought to investigate perlecan’s role in orchestrating early signaling events leading to precartilage condensation. According to our proposed model, we hypothesize that perlecan, a prominent component of embryonic mesenchyme tissue, plays a significant role in regulating precartilage mesenchyme cell-matrix interactions. Here, I will summarize key findings for this study.

1. A unique bioactive region within the forth domain of perlecan, PLN IV-3 consisting of Ig modules 16-22, was identified. For this assay, ATDC5 cells were cultured on various perlecan derived subdomains, substratum-induced morphological changes were used as measure of perlecan activity. Although, perlecan domain IV is composed of similar Ig modules, only PLN IV-3 produced a significant response.

2. As a substrate PLN IV-3 stimulated an immediate and robust response in ATDC5 cells. Within 1hr of seeding, cells began to form cell-cell adhesion over cell-substrate attachment. Previous studies have shown intact full-length perlecan bearing GAG side chains has antiadhesive properties, while the core alone promotes cell attachment. Our data provides contrasting results, in fact when tested against intact GAG bearing perlecan, PLN IV-3 had a much stronger cluster effect.
3. A mutant PLN IV-3 subdomain, harboring an R-to-Q substitution at residue 3452 and is associated with the skeletal disorder SJS, was shown to have reduced cluster activity.

4. In SJS patients, mutant perlecan products form inclusion bodies and are retained within the cell, indicative of protein misfolding and/or aggregation. The structural properties of PLN IV-3 and mutant R3452Q were compared by CD spectroscopy. I found no major conformational changes, although its likely R3452Q may increase protein aggregation with very little impact on overall structural.

5. CD thermal analysis revealed that perlecan domain IV constructs partially unfold at about 60°C. At higher temperatures the proteins are completely denatured. Heating PLN IV-3 also preventing protein aggregation, as visualized by AFM imaging.

6. Preheating PLN IV-3, prior to coating as a substrate, enhanced ATDC5 cell clustering. This is likely attributed to increased exposure of the active site. The R3452Q mutant had the same effect when heated and its net effect was the same as native PLN IV-3.

7. The PLN IV-3 induced cluster formation process begins with the disruption of focal adhesion points. In a cell adhesion analysis, PLN IV-3 prevented almost all cells from early substrate attachment (less than 10% attached). Furthermore, dephosphorylation of focal adhesion components FAK and SRC occurred, as demonstrated by western blot analysis using specific antibodies to target the active forms of each molecules.
8. PLN IV-3 mediated ERK, which is a major component of the MAPK signaling cascade, inactivation as demonstrated by western blot analysis. FoxM1, a downstream target of ERK, was reduced as well. Loss of ERK signaling coincided with decreased ATDC5 metabolic activity.

9. Reduced proliferation was associated with an increase in cell cycle inhibitors *cdkn1c* and *atf3*. These findings corroborate FoxM1’s role in cell-cycle progression. Here we establish a link between perlecan and the transcriptional activity of FoxM1.

10. PLN IV-3 increased expression of chondrogenic genes *sox9*, *col21*, and *acan*. However, in long term cultures PLN IV-3 led to a delay in total GAG production. This is likely a reflection of lower total number of cells. In this case QPCR is likely a more reliable indicator of matrix formation.

The focus of this study is to better understand perlecan’s role in skeletogenesis, with a keen interest in uncovering the function of the perlecan core protein during mesenchyme condensation. This stage of development is crucial for proper cartilage and bone formation, and coincides with high levels of perlecan deposition. This study concludes with the identification of a bioactive perlecan subdomain, PLN IV-3, with a demonstrated role in cell condensation and MAPK signaling. Here, I used an established *in vitro* chondrogenic cell model, ATDC5, to uncover novel features of the perlecan core protein. PLN IV-3 produced a striking effect on ATDC5 cells, where cells immediately responded by forming cell-cell interactions and developed into tight dense cell clusters comparable to mesenchymal cell condensation and cartilage nodules. As a whole the perlecan core protein generally favors cell adhesion and spreading, although PLN IV-3 seems to have
an opposing role (Grindel et al., 2014; SundarRaj et al., 1995; Whitelock et al., 1999).

Interestingly, a peptide located within PLN IV-3 is also known to promote cell attachment (Farach-Carson et al., 2008; Pradhan et al., 2009). As we know, the manner in which perlecan is presented can have differential effects at the cellular level. The most well-known case being the domain V derived product endorepellin, which has antiangiogenic properties, whereas a whole perlecan promotes angiogenesis (Lee et al., 2011; Mongiat et al., 2003). Domain IV at large may have one particular function, but its role may change upon exposure to certain active sites. Here, I have shown that PLN IV-1 and IV-2 are unable to produce the same response as PLN IV-3. A mutant R3452Q subdomain that differs by only one residue drastically reversed the cell cluster phenotype, without impacting the conformation of PLN IV-3. We discovered that when partially unfolded by heat PLN IV-3 activity was enhanced. The same was seen with R3452Q. This is likely due to increased exposure of the active signaling element.

The mechanism by which PLN IV-3 induces cell clustering begins with the interference of focal adhesion points. I have shown that both FAK and SRC are dephosphorylated upon ATDC5 cell contact with PLN IV-3 substrate. The mechanism of action begins with the blockage of cell-substrate attachment, which in turn forces cells to form cell-cell interactions. PLN IV-3 induced cell clustering is mediated by increased gene expression of N-cadherin and Sox9, two hallmarks of condensing cells (Bi et al., 1999; Oberlender and Tuan, 1994). As these nodule-like formations occurred, ATDC5 cells increased their gene expression of cartilage matrix components, collagen 2 and aggregan (Mallein-Gerin et al., 1988). PLN IV-3 activity appears to be coupled to MAPK signaling. Here, I demonstrated that PLN IV-3 stimulation decreased the phosphorylation of ERK1/2, a key
component of the MAPK signaling cascade (Ashraf et al., 2016). Thus, loss of ERK activity was accompanied by decreased ATDC5 cell metabolic activity. I validated this response by examining events downstream of ERK signaling. One of ERK’s targets is the transcription factor FoxM1, which controls cell cycle-dependent gene expression (Chen et al., 2013). For the first time, FoxM1 has been implicated in an in vitro cell precartilage condensation model. Control of cell proliferation regulates chondrocyte differentiation into hypertrophic chondrocytes (Akiyama et al., 2002; Bi et al., 1999). Sox9 is required at several successive steps of chondrocyte differentiation, and inhibits the transition of proliferation chondrocytes to hypertrophy. Furthermore, PLN IV-3 significantly increased the expression of cell cycle inhibitor genes cdkn1c and atf3.

4.2.1 Future directions

Future studies will seek to identify the biochemical interactions between PLN IV-3 and cell surface receptor(s). Given FAK and SRC are involved in PLN IV-3 activity we speculate integrin stimulation also plays a role (Beauséjour et al., 2012). In the classic integrin signaling model ligand-integrin binding activates FAK/SRC, but in our case we observed the opposite, where PLN IV-3 appears to block integrin-substrate recognition. Although rare, integrin stimulation has been show to decrease FAK activity (Kanazawa et al., 1995). Investigating the integrin-PLN IV-3 interaction is one avenue worth exploring. As described in appendix G, I have already developed a binding assay to measure these interactions. In addition, there are a number of well-established methods for inducing/inhibiting integrin stimulation at the cellular level. Valuable information could be gained by integrating these tools into our PLN IV-3 induced chondrogenic model. We must also keep in mind other mechanisms involved in the process. Because
of PLN IV-3 “repulsion” response, semaphoring/receptor complexes have gained our attention. Semaphorins function as a chemorepulsive agent and are important for cell attachment and migration (Jongbloets and Pasterkamp, 2014). Interesting, semaphorin family members encode a Ig-like C2type Ig domain, similar to those found in domain IV of perlecan (Inagaki et al., 1995). SEMA3F has been reported to deactivate α5β3 integrin, disrupt cell-substrate adhesion, and down regulate ERK1/2 signaling (Potiron et al., 2007). Semaphorin-3A and its receptors, neuropilin-1, and plexins A-1 and A-2, are expressed during endochondral ossification (Gomez et al., 2005). Semaphorin-3A is also expressed in osteoarthritic cartilage, where it interferes with endothelial cell migration by antagonizing the vascular endothelial growth factor 165 (Okubo et al., 2011). As discussed in the introduction (chapter 1 section 1.9.3), in drosophila perlecan (trol) facilitates transmembrane semaphoring-mediated repulsive guidance (Cho et al., 2012). As to date, semaphorin signaling in cartilage formation is virtually uncharted. Future studies should characterize semaphorin and its associated receptors in in vitro chondrogenic models. This study would be the first of its kind.

A further characterization of PLN IV-3 MAPK signaling in various cell models and an in vivo characterization of perlecan signaling is key to fully establishing this phenomenon as a meaningful mechanism for cartilage and bone formation. We have already began these studies as shown in appendix f. Fortunately, Dr. Farach-Carson has access perlecan deficient mice, which can be used to validate our in vitro findings and establish perlecan functionality in a pre-clinical animal model (Lowe et al., 2014; Thompson et al., 2011; Wang et al., 2014).
In the future I would like to see these findings incorporated into tissue engineering strategies. Although a required molecule for bone and cartilage formation, perlecan has remained largely ignored for such studies. In the last few years Dr. Farach-Carson’s lab has made great advances in the field of 3D cell culture technology ((Engel et al., 2015; Fong et al., 2015; Ozdemir et al., 2016). Her lab is well-experienced in cross-linking functional perlecan derived products into 3D cell culture systems. Similar methods could be applied to PLN IV-3, where PLN IV-3 is incorporated into the matrix scaffold to drive mesenchyme condensation and subsequent cartilage formation.
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Appendices

This appendices will summarize key findings not discussed in previous chapters that are organized around my publications but were significant to my dissertation work and overall graduate learning experience. Appendix A and B consist of supplemental figures that support the studies presented in chapters 2 and 3, respectively. Appendix C includes a collection of early bioinformatics work that led to the construction of the perlecan recombinant proteins. Appendix D list all perlecan domain constructs that I have created. The purpose of appendix C and D is to outline key details of the domain IV constructs for anyone wishing to express these fragments or modify their current state. Appendix E provides important information regarding the handling and storage of PLN-IV-3, which is currently the focus of two ongoing studies. Appendix F list all the cell lines that I have tested for PLN IV-3 activity. Appendix G and H consists of preliminary studies that I have performed in effort to identify the perlecan-osteocyte binding receptor. These studies will be continued by Drs. Farach-Carson and Wang, as part of a major collaborative project investigating perlecan’s role as mechanosensing tether at the osteocyte cell surface. Appendix I consists of a study I collaborated on with Dr. Peter Lwigale (Professor at Rice University) and his former graduate student Dr. James W. Spurlin (now at a post-doc fellow at Princeton University). The purpose of this study was to determine if AFM tissue stiffness characterization could be used to access matrix remodeling in the chicken cornea.
Appendix A Supplemental material for chapter 2

Purified heparan sulfate (HS) was used as a control to determine the possible effects of perlecans’s HS attachments in AFM force measurements. Similar to what we observed with full-length perlecans, individual HS were difficult to visualize. We found evidence supporting the HS chains of perlecans may in fact be contributing to protein clustering and it is likely why we often cannot distinguish the HS attachments from the perlecans core protein. Figure A.1 contains representative AFM images of purified HS. HS were also subjected to stretch-pulling and force curves were generated (figure A.2 A). We fitted the curves with a eWLC model and found they had a relatively low persistence length of 0.4 nm, which is much lower than we found with either full-length perlecans or the protein core itself (figure A.2 B. This supports perlecans’s HS chains a flexible extensions of the perlecans core protein. Furthermore, we showed the HS chains of domain I can be recognized by AFM imaging (figure A.3).
Figure A.1 AFM images of HS. Purified HS from bovine kidney was imaged by AFM to determine whether or not perlecans’s GAGs could be visualized by AFM (A) Concentrated HS (10 µg/ml) formed aggregates that made individual chains unresolvable. The clumping of HS may explain why perlecans’s chains were not seen in. (B) At a lower concentration (1 µg/ml) thin linear molecules were observed on occasion (left box), but most often small clumps (right box) were observed.
Figure A.2 AFM stretching of purified HS. To examine how perlecan’s GAG chains may influence mechanical for measurements, purified HS was subjected to AFM stretching. (A) Single force-extension curves for bovine kidney purified HS are shown on the left. (B) On the right are superimposed force-extension curves of HS chains. The eWLC fit to the HS data (black line) gives a persistence length of ~ 0.4 nm, much lower than that of full-length perlecan (1.2 nm) and the perlecan core (22 nm).
Figure A.3 AFM images of perlecan domain I. Domain I’s GAG side chains were visualized by AFM. In figure A several single monomers and a large complex were captured. Within this field of image the GAG chains of domain I (Dm I) are not visible. However, in images B and C, long linear structures are found attached to a core modular structure. Domain I contains three GAG attachment sites, but here, at most, one chain per protein core is observed.
Appendix B Supplemental material for chapter 3

In our chapter 3 studies we found that almost every cell immediately responded to PLN IV-3, but after a few days or so (depending on plating conditions, i.e. amount of substrate or cell seeding density) the cells appeared to overcome this response. One of our initial thoughts was that the ATDC5 cells were degrading the PLN IV-3 substrate and therefore the active signaling sequence was lost. We found this was not the case by performing western blots using a specific perlecan 3135 antibody that recognized full-intact PLN IV-3 (figure B.1). It is possible these cells have undergone phenotypic changes that make them no longer responsive or at this point they have produced their own matrix, which they can then use as a substrate to attach and spread on. Figure B.2 is data obtained from CD thermal measurements of PLN IV-3. In chapter 3 I showed that heating PLN IV-3 to 60°C partially unfolded the protein, but at higher temperatures the protein completely denatures and was no recoverable. I wanted to determine if PLN IV-3 could refold after 60°C treatment. Here, PLN IV-3 was heated to 60°C and cooled back down. I found that the protein fold partially recovered, although, according to CD spectra the protein conformation had been drastically affected.
Figure B.1 PLN IV-3 substrate remains stable over 48 hr. ATDC5 cells were cultured on PLN IV-3 coated plates for 0, 1, 2, 4, 12, 24, and 48 hr, at which point adsorbed PLN IV was collected and processed for western blot analysis. A domain IV specific antibody (3135) was used to detect intact PLN IV-3, demonstrating ATDC5 cells were not degrading the perlecan matrix.
Figure B.2 Far-UV CD spectral analysis of PLN IV-3. PLN IV-3 was unfolded at 60°C and cooled back down to 30°C to allow refolding, with spectra recorded at every 10°C. (A) Spectra for heated PLN IV-3 are shown on the left. When heated from 30°C (dark blue) to 60°C (yellow) the PLN IV-3 fold is lost, indicated by a shift in the minimum from 217 nm to 212 nm and a loss of the maximum at 202. (B) Shown on the right, when cooled back to 30°C there is a recovery of the minimum and restoration of the maximum, although the maximum does not fully recover. Results suggest on a partial recovery of the PLN IV-3 fold.
Biinformatics analysis of perlecan domain IV

Prior to my dissertation work, only a select few studies had investigated perlecan domain IV (Hopf et al., 2001, 1999; Kvansakul et al., 2001; Pradhan et al., 2009) Moreover, most studies experimented with perlecan derived products of mouse origin. Although sequences are very similar between human and mouse perlecan, the mouse protein lacks 7 of the Ig repeats found in human (Murdoch et al., 1992; Noonan et al., 1991). I began my graduate career constructing recombinant human perlecan subdomains to test for functionality, as told in chapter 3. To get there, a substantial amount of work went into the analysis of the perlecan protein sequence. These studies laid the foundation for many research advances. Here, I will summarize the sequence and structural analysis that led to the design of perlecan recombinants.

Given perlecan’s complex structural design and large size, I decided to compare sequence and structural properties of the individual Ig modules of domain IV. For sequence information the websites by GeneCards (HSPG2 ID = GC01M021822; http://www.genecards.org) and Uniprot (HSPG2 ID = P98160; http://www.uniprot.org) were valuable resources. Listed in table C.1 is the entire sequence of domain IV broken down into individually Ig modules 2-22. Each Ig sequence was modeled using the freely available online software PHYRE. PHYRE uses a template-based modelling method, where the user given amino acid sequence is aligned to a sequence of known protein structure and coordinates are adjusted accordingly to create a predicted 3D structure. The top three matches for each Ig module are listed in table C.2. All the top predicted matches share homology to one of five types of known protein structures (table C.3). Representative predicted structures for each type are shown in figures C.1-5. Protein data
bank (PDB) files were generated with PHYRE and processed with Visual Molecular Dynamics (VMD) software, which is available for download online (http://www.ks.uiuc.edu/Research/vmd). Although perlecan is composed of similar Ig folds, one must consider that even the slightest differences can lead to robust responses (Farach-Carson et al., 2008; Grindel et al., 2014; Pradhan et al., 2009). Domain IV, remains relatively unexplored but as we are finding out, has potential roles in many biological processes. Combining both bioinformatics and functional studies will be key to uncovering novel structures within the perlecan core protein.
Table C.1 Perlecan domain IV amino acid sequences listed by assigned number of Ig modules

<table>
<thead>
<tr>
<th>Ig module</th>
<th>Full sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>LVVEVHPARSIVPQGGSHVSLRCQVSGSFGYPYYSREDGRVPVSPGTQRRHGSQSLHFPSVQPSADGVYICTCRNLHQSNTSRAELLViquidSKPI</td>
</tr>
<tr>
<td>3</td>
<td>TVTVEEQRSQSVRPGADVTFCATAKSKSPAytLWVTWLHSLHCGLPTRAMDFNGILTIRNVQLSDAITYVCTGSNFAMIDQGATTATLHVQASGLSA</td>
</tr>
<tr>
<td>4</td>
<td>PVVSIHPQGTLTVQPGQLAEFRCSATGSTPTPLETWGPGQQLPAKQAEEHGLRLREPAFETPDQCAYLCLRAISSAQGQVARAHLHVQGGG</td>
</tr>
<tr>
<td>5</td>
<td>PRVQVSPERTQVHAGRVTLYCRAAGVPSAITTWKEGSLPPPQARSDTIDALTLPAPAATTADAGFYLCLVATSPAGTQAARIQVVLVSASDAPPP</td>
</tr>
<tr>
<td>6</td>
<td>PVKIESSSPSVTEGQLDLNCVAG8ASAIAHAQVTWYRGGSLPPHTQHVGSRLRLPQVSPADSGEYVCRVENGSIPKEASITVSVLHGTSHSIPSYTPVPGST</td>
</tr>
<tr>
<td>7</td>
<td>RPIREPSHSISHVAEGQTLDLNCVPGQHAQVTWHKRSGLPPRHQHTHGSLLLRLHQPVTADSGEYVCHVQSVTSGPLEASVLVTIESAVPGPI</td>
</tr>
<tr>
<td>8</td>
<td>PPVRIESSSTVAEGQTLDLSCVVAQQAHQVTWYKRGGSLPPRHQVGRSLYLIFQAPSAADAGQYVCRASNGMEASITVTVGTQAGNALAYPGST</td>
</tr>
<tr>
<td>9</td>
<td>QPIRIPSSSQQVAEQGTLDLNCVPGHQSHAQVTWHKRSGLPPRHQHTHGSLLLRLQASPDSAGYEYCRVLGSSVPLEASVLVTIEPASVPALGVT</td>
</tr>
<tr>
<td>10</td>
<td>PTVRIESSSHPAHRQSTLDLNCVPGQHAQVTWHKRSGLPPRHQHTHGSLLLRLQASPDSAGYEYCRVLGSSVPLEASVLVTIEPASVPALGVT</td>
</tr>
<tr>
<td>11</td>
<td>YVPRIESSSASLANGHTLNLVCASAPHTWTYWYKRGGLSPRSRHIVGSLRLPQVTPADSGEYVCHVQSVNAGSRTSLIVTIQGSSSHVPSVS</td>
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<tr>
<td>12</td>
<td>PPRIESSSPTTVETGQLDLNCCVAG8ASAIAHAQVTWYRGGSLPPHTQHVGSRLRLPQVSPADSGEYVCRVENGSIPKEASITVSVLHGTSHSIPSYTPVPGST</td>
</tr>
<tr>
<td>13</td>
<td>MPRIESSSSHVAEGQTLDLNCVPGQHAQVTWHKRSGLPPRHQHSGSLRLQASPDSAGYEYCRVLGSSVPLEASVLVTIEPASVPALGVT</td>
</tr>
<tr>
<td>14</td>
<td>PPRIRIPSSSRVAEQGTLDLKCVCVPGQHAQVTWHKRSGLPPRHQHSGSLRLQASPDSAGYEYCRVLGSSVPLEASVLVTIEPASVPALGVT</td>
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<tr>
<td>15</td>
<td>QPITVEAASSHSVETGQTLDLNCVPGQHAQVTWHKRSGLPPRHQHSGSLRLQASPDSAGYEYCRVLGSSVPLEASVLVTIEPASVPALGVT</td>
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<tr>
<td>16</td>
<td>PVISIPSSSTTVQGQDASIFCKLHGDAPISLWKKTRNQELEDNVHISPNGSITTVGTIRPSNHHTGRVCASNYVAQSVVNSLVHPP</td>
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<tr>
<td>17</td>
<td>TVSIPLQEGPVWVKAVTLCVSAGEPRSSARWTRISSPAPKLEQRTYGLMDASHAVLQISSAKPDSAGTYVCLAQNLGTAQKQVEVTDGAMAPGA</td>
</tr>
<tr>
<td>18</td>
<td>PQVQAAEEAWEAEGHTALLRCSATQHSGAPINTHWSLRLSPWQHLREQGDTLIIRRV AQVQGSGYQICNATSPAGHAEATITLHVSEP</td>
</tr>
<tr>
<td>19</td>
<td>PYYATTVPEASHVQAGESTVQQLCLAHHTPTLQFQWVQSSGLPTRATARNELLHFFERAPEDSGYRRCRVTNKVGSAEFQAQLLVQGPPSGLPASIPAGST</td>
</tr>
<tr>
<td>20</td>
<td>PTQVQVTEQGKSGAAESEFCAYTSVQGQPDSKVLDDGSLPPDSRLENNMLMLPSVRPQDAGTVYCTATNRQGQKVAFAHLQVPFS</td>
</tr>
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<td>21</td>
<td>VLINRSTQVTVVGVHAVEFELALGDPKPQTVWSKVQGHLRPPGQVSVQGVRVIALHVELADAGQYRCTATAAGTTQSVSHLLVQVA</td>
</tr>
<tr>
<td>22</td>
<td>LPQISMPQEVQPSVAHSAVFPSLASQYPPTDPCLSLQRLDDGSLPPDSRLENNMLMLPSVRPQDAGTVYCTATNRQGQKVAFAHLQVPERV</td>
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</table>

Ig module 1 is located within domain II and therefore not included in this list. Domain IV begins with Ig module 2.
Table C.2 PHYRE predicted structures for individual Ig modules

<table>
<thead>
<tr>
<th>Ig module</th>
<th>First match (SCOPE ID, protein lineage)</th>
<th>Second match</th>
<th>Third match</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>c2dm3a, KIAA0992 protein.paladin*</td>
<td>c2edjA, roundabout homolog 2</td>
<td>c2eo9a, roundabout homolog 1</td>
</tr>
<tr>
<td>3</td>
<td>D1a4b, Perlecian Ig3 domain, mouse*</td>
<td>D1cs6a4, Axonin-1 from Chicken*</td>
<td>c2edjA</td>
</tr>
<tr>
<td>4</td>
<td>d1cs6a4</td>
<td>D1bioa4, Hemolin from Moth</td>
<td>c2edjA</td>
</tr>
<tr>
<td>5</td>
<td>D1koaa1, Twitchin from Nematode *</td>
<td>c2dm3a</td>
<td>D2eqlA, Telokin from Human</td>
</tr>
<tr>
<td>6</td>
<td>d1cs6a4</td>
<td>d1koaa1</td>
<td>d2eqlA</td>
</tr>
<tr>
<td>7</td>
<td>d1koaa1</td>
<td>d1cs6a4</td>
<td>d2eqlA</td>
</tr>
<tr>
<td>8</td>
<td>C2eo9a, fifth Ig-like domain from human</td>
<td>d1cs6a4</td>
<td>d2eqlA</td>
</tr>
<tr>
<td></td>
<td>Roundabout homolog 1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>d1cs6a4</td>
<td>d1koaa1</td>
<td>c2edjA</td>
</tr>
<tr>
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<td>d1koaa1</td>
<td>c2dm3A</td>
</tr>
<tr>
<td>11</td>
<td>c2eo9a</td>
<td>cdm3a</td>
<td>d1koaa1</td>
</tr>
<tr>
<td>12</td>
<td>d1cs6a4</td>
<td>d1biha4</td>
<td>c2r3A, fourth Ig-like domain from myosin light chain kinase, smooth muscle</td>
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<tr>
<td>13</td>
<td>c2eo9a</td>
<td>c2dm3a</td>
<td>d1cs6a4</td>
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<tr>
<td>14</td>
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<td>d2eqlA</td>
<td>d1koaa1</td>
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<td>d1cs6a4</td>
<td>c3bf0c, g-like C2-type 2 domain of the human Mucos-associated lymphoid tissue lymphoma translocation protein 10</td>
<td>d1koaa1</td>
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<tr>
<td>16</td>
<td>d1cs6a4</td>
<td>d1koaa1</td>
<td>c2edjA</td>
</tr>
<tr>
<td>17</td>
<td>c2dm3A</td>
<td>c2dm2a, first Ig domain of human palladin</td>
<td>d2eqlA</td>
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<tr>
<td>18</td>
<td>d1cs6a4</td>
<td>d1koaa1</td>
<td>D1ncea, Titin from Human</td>
</tr>
<tr>
<td>19</td>
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<td>c2eo9a</td>
<td>d1koaa1</td>
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<tr>
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<td>D1cs6a3, Axonin-1 from Chicken</td>
<td>d1ncea</td>
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<td>d1cs6a4</td>
<td>d1ncea</td>
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</tr>
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</table>

* Corresponds to one of five first matches for all 22 Ig modules
Table C.3 List of top matched structures for Ig modules 2-22

<table>
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<tr>
<th>Type</th>
<th>SCOPE ID</th>
<th>Source</th>
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<tr>
<td>1</td>
<td>c2dm3a</td>
<td>Ig module human palladin</td>
</tr>
<tr>
<td>2</td>
<td>d1gl4b</td>
<td>Ig module mouse perlecan</td>
</tr>
<tr>
<td>3</td>
<td>d1cs6a4</td>
<td>Ig module axonin 1 chicken</td>
</tr>
<tr>
<td>4</td>
<td>d1koaa1</td>
<td>Ig module twitchin nematode</td>
</tr>
<tr>
<td>5</td>
<td>c2eo9a</td>
<td>Ig module roundabout homolog 1</td>
</tr>
</tbody>
</table>

Based on PHYRE homology modeling, the Ig modules of domain IV (Ig 2-22) match to one of the five types of Ig structures listed above.
Figure C.1 Perlecan Ig module 2. Based on PHYRE predicted modeling the Ig module 2 is similar to the human palladin Ig fold, listed as type 1 in table C.3. An α-helix is shown in blue and β-sheets are represented by yellow arrows.
**Figure C.2 Perlecain Ig module 3.** The predicted 3D structure of the human Ig module 3 best matched to that of the mouse perlecain Ig3 fold, listed as type 2 in table C.3. The structure of the mouse Ig3 fold was solved as a complex with nidogen-1 and is listed in the protein data bank (PDB) archive under ID: LG14.
Figure C.3 Perlecan Ig module 4. Based on sequence and predicted structural analysis, the Ig module 4 of domain IV favors the type 3 fold, or axonin 1 of chicken (table C.3). β-sheets are represented by yellow arrows.
Figure C. 4 Perlecan Ig module 5. Ig module 5 matches the type 4 3D predicted model. This type of fold is found in twitchin of nematode (table C.3). β-sheets are represented by yellow arrows.
Figure C.5 Perlecan Ig module 8. The Ig module 8 of domain IV favors the 3D model that matches to type 5, an Ig fold roundabout homolog 1. Yellow arrows indicate β-sheets.
Appendix D Perlecan domain IV constructs

In addition to the subdomains described in chapter 3, a domain IV-3 MMP7 cleaved product was cloned, known here as LEQ. Future studies will investigate the role of LEQ and other perlecan-derived products in cancer discohesion and invasion. This fragment was identified through N-terminal sequencing, Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS), and liquid chromatography MS (LC-MS) analysis of Mmp7 digested IV-3 (Grindel et al. 2014). LEQ (Ig repeats 17–20, amino acids 3156–3480, base pairs 10631–10480), PCR products were amplified with a forward primer containing an XbaI sequence and a reverse primer flanked with an Age1 site, and inserted into the PLN IV-3 expression vector. The remaining procedures can be found in Grindel et al. (2014). Here, I wanted to provide a list of all the perlecan domain IV constructs and highlight elements that will be help for future manipulation if desired. Figures D.1-4 were created using the bioinformatics software Geneious v 5.4 and show the entire amino acid sequence for all constructs (Kearse et al., 2012). Stable HEK293 cells lines expressing each perlecan fragment were created and a reliable protocol was developed to isolate and purify these perlecan subdomains (Grindel et al., 2014).
Figure D.1 Perlecan subdomain PLN IV-1 amino acid sequence. Protein sequence for recombinant domain IV-1. Includes cleaved BM40 sequence up to the C-terminal enterokinase (EK) cleavage site and His tag. PLN IV-1 is shown in yellow. Important restriction sites Nhe1 and Not1 are included as points of reference and to highlight ease of construct manipulation.
**Figure D.2 Perlecan subdomain IV-2 amino acid sequence.** IV-2 was inserted into domain IV-1 vector and is shown in yellow. All regions flanking domain the perlecan region are the same as IV-1 vector.
Figure D.3 Perlecan subdomains IV-3 and R3452Q amino acid sequence. Protein sequence for recombinant domain IV-3. Perlecan is shown in yellow and the SJS mutation R3452Q is highlighted in green. Important restricting sites Xba1 and Age1 are included as points of reference and to highlight ease of construct manipulation. Immediately flanking IV-3 is the EK, followed by a linking region, and then the His tag.
Figure D.4 MMP7 produced LEQ fragment. LEQ represents a smaller fragment with domain IV-3. An extra EK site was included between the BM-40 signal sequence and start of LEQ. In addition the linking region between the EK site and His tag were removed.
Appendix E PLN IV-3 aggregation

As shown in chapter 3, PLN IV-3 played a major role in my dissertation. However, one of the issues with PLN IV-3 was that it aggregated when stored as an isolated protein in solution. This is not surprising considering that Ig molecules are naturally “sticky”. Aggregation became apparent immediately following purification, when precipitation was first observed. Subsequent experiments later confirmed this was the case. This is a major problem that decreased production yields and negatively impacted experimental results. Structural analysis are difficult, if not impossible, to perform using protein aggregates. For these reasons I became determined to find ideal conditions that would preserve the single monomer form of PLN IV-3 for both structural and functional studies.

Protein aggregation was observed while attempting to perform AFM studies for chapter 3. Shown in chapter figure 3.6 A is an image captured by AFM in which PLN IV-3 protein aggregates could be seen. We later discovered that by applying heat, we could break apart these aggregates into monomers, as seen in chapter 3 figure 3.6 B. In addition, when separated on a size exclusion column most of PLN IV-3 eluted off as a large complex within the void volume (figure E.1). There also appeared a small peak corresponding to the molecular weight of a single monomer, but this was a small fraction of the total sample. We now know that protein structure is not compromised until a temperature of 60ºC is reached. For future studies it is recommended best to apply heat if applicable to the experiment, as was done in cell culture studies chapter 3.

With PLN IV-3 being used for various studies, it was important to find a storage buffer appropriate for all assays. I developed a method in which I could screen through many
conditions at once. At the end of Ni-NTA purification, perlecan was eluted in a 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 200 mM imidazole, and 0.05 % (v/v) Tween-20 at pH 8.0, following Qiagen’s QiaExpressionist protocol. PLN IV-3 containing fractions were pooled together and dialyzed into a variety of buffers (listed at the top of table E.1). In addition, various supplements were added to help with solubility (listed at the bottom of table E.1). Denaturing agents were added as controls. From here, protein solutions were passed through a 100K MW membrane cutoff by centrifugation following the Amicon Ultra protocol (EMD Millipore). Proteins were centrifuged at 4,000 g for 5-10 mins. This allowed for soluble IV-3 monomers (75 kDa) to pass through the membrane. If solution favored aggregation then protein remained in top reservoir. A simple schematic is shown in figure E.2. Both the soluble and aggregated forms were collected in equal volume. Solutions were processed to determine if buffer conditions induced aggregates or solubilized protein. Protein was detected by SDS-PAGE (silver stain) and by dot blot analysis using the anti perlecan domain IV antibody 3135. Samples of both detection methods are shown in figure E.1. The comments column lists key findings discovered throughout the process of purification and subsequent soluble/aggregation studies.

Current and ideal storage/usage conditions are indicated by (*). At present, the preferred storage buffer is one with HEPES (buffer no. 5, table E.1). Glycerol slightly helps but it is recommended it only be used for storage of frozen samples. Generally, most buffers are acceptable as long as Tween-20 is present, but I would not recommend long term use in buffers no. 1-4. If stored with Tween, then sample can be diluted into any of the buffers for short term use. These findings should serve as a guide for handling and using
perlecan domain recombinants. It is important for the user to ultimately decide which conditions will work for their system and adapt accordingly.
Figure E.1 PLN IV-3 aggregation. Size-exclusion chromatography of concentrated perlecan (1mg/ml) separated at 4°C. The graph represent absorbance vs retention time. Two peaks corresponding to different molecular weight forms of PLN IV-3 are observed. The majority of PLN IV-3 forms a large complex and runs out with void volume while a small peak of 75 kDa is observed and is consistent with the size of a single PLN IV-3 monomer.
### Table E.1 Buffers used to test PLN IV-3 solubility

<table>
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<tr>
<th>No.</th>
<th>Buffer</th>
<th>Comments</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$ (PBS)</td>
<td>Precipitates out of solution; use short term; avoid freeze-thaw</td>
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<tr>
<td>2</td>
<td>50 mM Na$_2$HPO$_4$, 300 mM NaCl</td>
<td>Precipitates out of solution; use short term; avoid freeze-thaw</td>
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<td>3</td>
<td>20 mM Na$_2$HPO$_4$, 100 mM NaCl</td>
<td>Precipitates out of solution; use short term; avoid freeze-thaw</td>
</tr>
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<td>50 mM Na$_2$HPO$_4$</td>
<td>Precipitates out of solution, use short term, avoid freeze-thaw</td>
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<td>5</td>
<td>25 mM HEPES, 50 mM NaCl*</td>
<td>Current storage buffer; used for ATDCS coating; ideal protein concentration is 1 mg/ml; protein crashes out over time and at high concentrations</td>
</tr>
<tr>
<td>6</td>
<td>H$_2$O</td>
<td>Used for CD analysis, okay for low protein concentration and short term use, do not use for storage</td>
</tr>
</tbody>
</table>

### Supplement

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<td>Dimethyl sulfoxide (DMSO)</td>
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<td>8</td>
<td>1M GdmCL</td>
<td>Protein aggregates</td>
</tr>
<tr>
<td>9</td>
<td>5-10% Glycerol*</td>
<td>Partially solubilizes protein; add for long term -80°C storage</td>
</tr>
<tr>
<td>10</td>
<td>0.05-0.5% Octyl glucoside</td>
<td>Protein aggregates</td>
</tr>
<tr>
<td>11</td>
<td>1% SDS</td>
<td>Protein aggregates</td>
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<tr>
<td>12</td>
<td>0.01-0.05% Tween 20*</td>
<td>Solubilizes protein in any buffer; if kept in stock/storage buffer then protein can be diluted into any non-tween buffer for short term use; best to include if applicable with experiment</td>
</tr>
<tr>
<td>13</td>
<td>0.5 M Urea</td>
<td>Protein aggregates</td>
</tr>
</tbody>
</table>
Figure E.1 Determining the optimal buffer for PLN IV-3 storage and usage. Following Ni-NTA purification, pooled column fractions were dialyzed and concentrated into various buffers (see table 5.1 for complete list with corresponding buffer No.) to remove imidazole. Samples were then placed into centrifuge tubes containing a 100 kDa pore size membrane, allowing for domain IV monomers (75 kDa PLN IV-3) to pass through. Upon centrifugation, soluble monomers (S) were collected in the filtrate receiver, while large aggregates (A), unable to pass through the membrane, remained in sample reservoir. Protein samples were screened by either SDS-PAGE (silver stain) or dot-blot to determine the best solvent for PLN IV-3. Buffer compatibility was determined by whether or not PLN IV-3 formed A or S. The two images shown in step 4 are examples of the results obtained by this assay. Above the silver stain images is the protein sample # (1-5) assigned to a particular buffer condition (listed by no. according to table E.1). Supplements added to the buffer are listed after the buffer no., e.g. “2,12”. A change in buffers is represented by “∆”. The two screening methods were used to determine buffer compatibility.
Appendix F PLN IV-3 activity in various cells types

In chapter 3, I specifically looked at PLN IV-3 in the context of chondrogenesis, using a prechondrogenic cell line ATDC5. However, IV-3 activity was tested in various cell lines and even primary cells isolated from mice (See table F.1 for list of all cells tested and their relative strength of response). ATDC5 appeared to have the greatest response in terms of cluster formation, while there was no morphological changes seen in the MLO-Y4 osteocytes cells. On the other hand, the osteoblast-like MC3T3-E1 cells did respond but not to the same extent as did ATDC5. Rather, the MC3T3-E1 cells formed a reticular like structure with void spaces, shown in figure F.1. Even so, FAK activation was still suppressed. These cells responded similarly to BM-MSCs, as mentioned in Chapter 3. Whether or not the same signaling mechanisms are involved in these responses remains to be seen.
Figure F.1 MC3T3-E1 cells respond to PLN IV-3. Mouse pre-osteoblastic cells MC3T3-E1 cells were cultured for 4 hr on standard tissue culture plates (A) or perlecan domain PLN IV-3 (B). When cultured on PLN IV-3, cells formed a reticular like structure. Images were captured in bright field using 10X objective. (C) Western blot analysis shows PLN IV-3 decreased FAK phosphorylation. Western blot protocol is described in chapter 3. β-actin (Abcam; catalog no. ab8229) was used as the load control for this assay.
Table F.1 PLN IV-3 activity in various cells

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<tr>
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<th>Strength of response</th>
<th>Notes</th>
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<td>ATDC5</td>
<td>+++</td>
<td>Form tight dense clusters</td>
</tr>
<tr>
<td>BM-MSCs</td>
<td>++</td>
<td>Temporary cell-cell adhesions</td>
</tr>
<tr>
<td>MC3T3-E1</td>
<td>+</td>
<td>No clustering; reticular-like network formed</td>
</tr>
<tr>
<td>MLO-Y4</td>
<td>-</td>
<td>No response</td>
</tr>
</tbody>
</table>

+++ Strong cluster phenotype; - No response
Appendix G PLN IV-3 binding assay

As Chapter 2 mentioned, it is of great interest to identify the perlecan receptor involved in mechanosensing. In an effort to do so, I worked on developing an assay for which we could screen candidate binding targets and I also examined the gene expression of candidate receptors in osteocyte cells.

Cell adhesion molecules (CAMs) play an important role in sensing and signaling (Wai Wong et al., 2012), although, these molecules remain undiscovered in osteocytes. In bone, the distribution of CAMs is not well described, although NCAM was found to be transiently expressed in osteoblasts during endochondral and intramembranous ossification (Behrens et al., 2012; Inoue et al., 2013).

Domain IV, the largest domain, serves as a likely candidate to bind to cell surface protein given it is composed of 21 tandemly linked Ig modules and is involved in multiple interactions with other matrix components that express Ig modules such as CAMs. It is well accepted that Ig modules bind to one another in heterotypic or homotypic interactions at cell surfaces (Shimono et al., 2012; Wai Wong et al., 2012). The binding between CAMs and heterotypic Ig modules is typically of lower affinity (<100-200nM, but can be stabilized, “zipper-like” by the multi-valency of tandem Ig repeats (Knox et al., 2001; Koper et al., 2012; van der Merwe and Barclay, 1994). With this in mind, I wanted to develop a strategy which I could use as a reliable screen for perlecan binding targets. I decided to perform solid-phase binding assays, which involved coating target proteins to a substrate and probing with perlecan recombinant fragments. For these studies, I needed to optimize the best conditions for detection.
Although many alternative methods were attempted, described below is the protocol that has generated the most promising data to date. I directly labeled perlecan domain IV-3 with biotin, either through primary amino (-NH$_2$) or sulfhydryl (-SH-2) groups (Thermos Scientific; cat. no. 21327 and 21900), or with a fluorescent dye 5 - (and - 6) – carboxytetramethylrhodamine, or 5(6) TAMRA (Anaspec; cat. No AS-81120) which also binds to -NH$_2$. I then tested the binding of labeled perlecan domain IV-3 to NCAM2, NrCAM, and EpCAM (R&D Systems; cat. no. 7729-CM-050, 2034-NR-050. 960-EP-050) recombinant ectodomains. All CAMs were recombinant human ectodomains fused with the Fc region of antibodies. For dot blot adhesion assays, 1 µg (in 40 µl of PBS) of recombinant NCAM2, NrCAM, or EpCAM was adsorbed onto a 0.45 µM nitrocellulose membrane for 2 hr at room temperature. PBS (blank), BSA, and rabbit IgG were used as controls. Membranes were blocked in 1% (w/v) non-fat milk in TBS-T for 3 hr at RT. Membranes were incubated with either biotin/TAMRA labeled IV-3 or Protein A control (Sigma, cat. no P3838) at 50µg/ml in a 0.1% (w/v) non-fat milk blocking solution, overnight at 4°C. Protein A was used as a positive control to recognize the Fc portion of each fusion protein and the rabbit IgG. The following day unbound fragments were removed by washing the membranes 3 X 5 mins in TBS-T solution. Biotin labeled proteins were detected by either streptavidin-HRP (1:200,000) in 0.1% (w/v) non-fat milk blocking solution for 1 hr, followed by a 5 min incubation with the ECL substrate, and then exposed to film. TAMRA-labeled molecules were directly measured for fluorescence (Abs/Em = 541/565 nm) with Kodak Imager. Similarly, screening was performed in a plate reader format. CAMs were adsorbed onto 96-well ELISA plates
following similar conditions and probed with TAMRA labeled PLN IV-3 and Protein A as a control.

Preliminary results are shown in Figure G.1 and represent a “successful” attempt. The dot blot layout scheme is depicted at the top of the figure with wells labeled according to the adsorbed binding target. PLN IV-3 binding was detected for all CAMs tested, as shown in top blot. As expected, no signal was detected for BSA and rabbit IgG. Protein A controls are shown below.

These results are promising and will serve as a guide for future studies. Although binding was achieved, this method calls for improvements to increase assay specificity and sensitivity. There are a few particular concerns that I must address. For one, I found that the labeling itself may interfere with binding. In the future it is likely to best perform binding studies with few, if any, modifications to perlecan. In this case, labeling PLN IV-3 through the only available cysteine (–SH) may be the preferred choice. Another issue is the aggregation of PLN IV-3 as previously mentioned in chapter 3 and Appendix E.
Figure G.1 Perlecan domain IV-3 binds to CAMs. Dm IV-3/CAM interactions were tested in a dot blot adhesion assay. Nitrocellulose-bound targets, shown in the top layout, were probe with biotin-labeled IV-3 and Protein A (positive control). IV-3 bound to NCAM2, NrCAM, and EpCAM (detected by streptavidin-HRP).
Appendix H Osteocyte cell-surface and adhesion molecules

Future studies will examine the interaction between perlecan and osteocytes. Currently, integrins containing β1 and β3 are considered the primary cell surface sensors in human osteocytes (Phillips et al., 2008; Thi et al., 2013). In general osteocyte surface adhesion molecules are not well characterized. To launch the investigation of the perlecan-osteocyte interaction, I profiled the expression of osteocyte cell adhesion genes by performing a PCR array (Qiagen; Cat. No. PAMM-013Z). For this assay RNA was obtained from osteocytes isolated from the tibia of five 24 week old mice following manufactures protocol. All procedures involving mice were approved by the IACUC of the University of Delaware. Table H.1 lists all the genes examined and levels of transcript expression relative to β-actin control.
### Table H.1 Genes analyzed by PCR array

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<th>Symbol</th>
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Appendix I Cornea AFM study

This work was performed in collaboration with Dr. Lwigle’s lab group. His lab focuses on the development and regeneration of the cornea. They developed a chicken model to examine key events that regulate the differentiation of the neural crest cells, and also study vascular and nerve patterning of the eye (ref 2480979 and 20811061 and 1553176). Our connection was established based on our mutual interest in ECM remodeling of developing tissues. Prior to our study, Dr. Lwigle and his group discovered that wounded chicken embryo corneas exhibit nonfibrotic regeneration (ref 24003085). We therefore wanted to determine whether the physical properties of the regenerating matrix were restored as well. To do so we combined AFM nanoindentation with immunohistochemistry analysis of various ECM proteins involved in wound healing. This would allowed us to accurately map tissue stiffness with the onset of matrix production as the tissue is remodeling.

Corneal tissue was processed as described in (ref 24003085), with the exception that both paraformaldehyde (PFA) and methanol were used for fixation. All other conditions were kept the same. Tissue sections at 10 µm thick were mounted onto glass slides to allow for immune-directed AFM probing. Data was collected on a Bioscope Catalyst, provided by the Houston Methodist Research Institute, and was equipped with an IX81 Olympus inverted confocal. AFM measurements were performed in contact mode in PBS solution with a MLCT cantilever having a spring constant of 0.01 N/m (probe C). Cantilevers were calibrated prior to each experimental run by the thermal tuning method provided by the Nanoscope Analysis software. Force curves were fit with a Hertz-Sneddon model and the Young’s modulus values were obtained.
Preliminary results are provided in figures I.1-4. Figure I.1 A is an example of section of embryonic day 10 control tissue (E10C) with its various layer labeled to show were force data was collected. Figure I.1 A is an E10C tissue that has been stained with laminin to identify the basement membrane. Figure I.1A is an E10 wounded (W) sample that shows increased fibronectin staining around the site of injury. Because sample preparation is such an important component of any AFM study, we wanted to improve our sample preparation protocol. We found that we received better probe-tissue contact when we switched to methanol fixation over PFA, and the overall tissue quality improved as well. This lead to decreased sample variability in our data (figure I.2). We found that we could accurately measure differences between the tissue layers. The Young’s’ modulus are shown for each tissue layer in figure I.3. When we looked at various stages of development (E7 – E19), we found that the tissue stiffened as it matured (figure I.4). From these preliminary studies we concluded that we have established a method for monitoring the physical properties of the developing cornea. We have characterized the tissue stiffness of normal tissue during E7-19 of development. The next step is to evaluate the tissue mechanics of regenerated tissue after wounding. Although the chick cornea appears to regenerate scar free it remains to be seen if the ECM physical properties are maintained. If our hypothesis is correct we should see the mechanical properties of the ECM restored at the site of the wound.
**Figure I.1 Cornea tissue layer organization.** Cornea tissue was isolated from chicken embryos at various stages of embryonic development (E 7-19) and subjected to AFM nano-indentation to measure tissue stiffness. For this study control tissue was compared to wounded tissue. (A) The corneal layers targeted for AFM probing are shown on a bright field image of a E10 control tissue sample (C). (B) Accurate and precise AFM force measurements were obtained via “point and shoot” and immuno-directed guidance. (B) The basement membrane was revealed by laminin staining (green). An intact basement membrane is represented by continuous green layer. (B) However, when wounded (W) the basement membrane is disrupted and therefore we used fibronectin to stain the underlying regenerating stroma region.
Figure I.2 Optimizing cornea tissue fixation protocol for AFM studies. Cornea tissue was fixed with either 4% paraformaldehyde (PFA) or 100% methanol. AFM force measurements were captured within the stroma region and resulting force data was used to compare to tissue quality. Overall methanol fixed tissue was more intact and provided a smoother surface for probing. The Young’s modulus were similar between the two fixation methods, although, PFA produced a larger variation. For these reasons methanol fixation was as chosen as the preferred method for our AFM studies.
Figure I.3 Comparison of corneal layer stiffness. Elastic modulus values are reported for each layer, beginning from the anterior epithelial layer to posterior endothelium. The epithelial layer and the underlying basement membrane (BM) were of equal stiffness and much higher than the other tissue layers. There was no difference observed between the various depths of the stromal layer, i.e. anterior stroma (AS), middle stroma (MS), and posterior stroma (PS). The Descemet’s basement membrane and endothelium are much less stiffer than their anterior counterparts.
Figure I.4 Characterization of normal cornea tissue stiffness during embryonic development. AFM was used to measure tissue stiffness of the basement membrane, anterior stroma, and posterior stroma layers during developmental stages E7-E19. Tissue was collected at days E7, E8, E10, E15 and E19 for analysis. The general trend for all layers tested is that as the tissue matures so does the Young’s modulus.