AN ABSTRACT OF THE THESIS OF

Stephanie K. Grissom for the degree of Master of Science in Comparative Health Sciences presented on December 18, 2018

Title: Role of Cartilage and Bone Matrix Regulation in Early Equine Osteochondrosis

Abstract approved:

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Stacy A. Semevolos

Abstract: Osteochondrosis (OC) results from disruption of the normal endochondral ossification process during articular cartilage and epiphyseal bone growth. Previous studies have revealed that cartilage canals are often associated with early OC lesions, especially along the ossification front. In early OC, extracellular matrix signaling pathways are expected to be altered, as they control matrix production and degradation associated with endochondral ossification. The aim of this study was to obtain a greater understanding of the role of cellular regulatory mechanisms in the pathogenesis of early equine osteochondrosis by identifying differences in gene and protein expression in both normal and diseased cartilage of adolescent horses. The objective of the study was to define the expression of chondrocyte signaling pathways in two specific regions, the osteochondral junction and in cells surrounding the cartilage canals. The hypothesis of this study was that when evaluating gene and protein expression of chondrocytes in diseased and normal early osteochondrosis samples, it was expected to find an upregulation of matrix metalloproteinases and a decrease in extracellular matrix gene expression within the osteochondral junction and cells surrounding the cartilage canals.

Samples were previously harvested from the femoropatellar joints of foals ranging in age from 1 to 6 months. Archived paraffin-embedded osteochondral samples (6

osteochondrosis affected, 8 normal controls) and cDNA from chondrocytes captured with laser capture microdissection (4 OC, 5 normal controls) were used in this project. Quantitative real-time PCR was then performed on pre-amplified samples, evaluating 18 target genes from extracellular matrix signaling pathways. The genes included Collagen types I, IIB, and X, Aggrecan, Aggrecanase-1, MMP-1, MMP-3, SOX 5, SOX 9, Jagged 2, COMP, Runx2, Gremlin 1, Ephrin B2, Lubricin (PRG4), Osteocalcin (BGLAP), bFGF, and TGF-ß3. Immunohistochemistry was performed on paraffin-embedded osteochondral samples for osteocalcin, Sox-9, and collagen type IIB using mouse monoclonal α-human antibodies and for lubricin using rabbit polyclonal α-human antibodies.

Downward trends were noted in gene expression of collagen type IIB, aggrecan, and SOX-9 in chondrocytes surrounding the cartilage canals of early osteochondrosis-affected samples compared to normal samples. In chondrocytes along the osteochondral junction, there was a trend of downregulation of lubricin and collagen type IIB in osteochondrosis samples compared to normal samples. No difference in osteocalcin, lubricin, collagen type II B, or SOX9 protein expression was apparent between OC and normal cartilage samples.

Osteochondrosis is a multifactorial developmental disorder with a complex system of closely regulated signaling pathways. Recent studies have explored the early pathogenesis of osteochondrosis, with particular focus on failure of vascular anastomoses at the cartilage canals, alterations in biomechanical forces around the cartilage canals, and regulation of extracellular matrix pathways. An aim of the current study is to extend this knowledge by evaluating expression of components of the extracellular matrix in both normal and osteochondrosis-affected adolescent osteochondral specimens. Results of the

current study indicate that several matrix genes may be downregulated, including collagen type IIB, aggrecan, Sox-9, and lubricin in osteochondrosis.

Limitations of this study include a targeted rather than comprehensive evaluation of genes expressed in the extracellular matrix. In addition, only two cell populations were studied due to having a limited time to complete the laser capture procedure prior to sample degradation. It is also important to note that gene and protein expression studies do not address longitudinal changes or activity of molecules within the region. The results of this study contribute to the overall understanding of the pathogenesis of osteochondrosis and aid in identifying the role of cartilage and bone matrix regulation in early equine osteochondrosis.

Role of Cartilage and Bone Matrix Regulation in Early Equine Osteochondrosis

by
Stephanie K. Grissom

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Major Professor, representing Comparative Health Sciences

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Dean of the College of Veterinary Medicine

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Stephanie K. Grissom, Author

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Jesus, you are the bright and morning star. There is no one like you. You are my strength and all of my hope is in you. All things are possible with you, and nothing is possible without you. Your relentless love has saved me. Through the hardest of times you are with me. Your love never fails, never ceases, never tires. May my life and all that I do bring you glory and honor because I love you so dearly.

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DEDICATION

For Gabe, my best friend and my solid rock.
God chose you to pick me up when I was down, to push me further when I felt like giving up, to bring me laughter when I was fighting back the tears, and to feel His love in the most tangible of ways. I'm so glad it was you.

Role of Cartilage and Bone Matrix Regulation in Early Equine Osteochondrosis

Chapter 1: Introduction

Osteochondrosis is a well-recognized disorder across multiple species that involves a disruption within the process of endochondral ossification during periods of growth (Semevolos, 2017). In horses, this manifests clinically as articular cartilage flap defects in predilected sites with or without involvement of the subchondral bone. These lesions adversely affect the animal by creating inflammation within the joint and may be paired with clinical lameness. The impact on the equine industry is significant due to reduction in value of the animal and loss of use for athletic and breeding purposes. The current standard involves diagnosis after onset of clinical signs followed by arthroscopic surgical removal of affected tissue in order to reduce degenerative joint effects and pain (Mcilwraith, 2013). The majority of research in this area has focused on the complex etiology of clinical disease which involves nutrition, biomechanics, and genetics. Recently, focus has been redirected towards characterizing the pathogenesis of early osteochondrosis prior to clinical manifestation of disease (Laverty and Girard, 2013).

The purpose of this study was to further characterize molecular events that occur during critical periods of growth. Using real time qPCR and immunohistological staining, gene and protein expression were evaluated and findings compared between foals with and without evidence of early osteochondrosis in archived samples. The samples were previously processed using laser capture microdissection in order that two sites of interest could be evaluated, the osteochondral junction and cells surrounding the cartilage canals. Our hypothesis was that cellular regulatory events would result in differences in gene and protein expression between foals with and without disease. More specifically, it was expected to find a downregulation of extracellular matrix gene and protein expression and an upregulation of matrix degradation molecules within the osteochondral junction and in

cells surrounding the cartilage canals of early osteochondrosis affected samples. These effects may contribute to a weakened extracellular matrix causing susceptibility to shearing forces at these specific sites, creating focal areas of chondronecrosis which ultimately result in clinical disease.

The Equine Joint

The articular structures face a unique challenge in that they must meet requirements that demand strength and rigidity as found in bone while concurrently providing a level of flexibility and resiliency to dissipate forces created through equine locomotion (Mcilwraith, 2016). In addition, the articular surface must provide smooth, frictionless motion that is adapted to both routine and strenuous athletic activity. A disruption of one or more of these seemingly small components profoundly affects the system as a whole and may result in instability, pain, and loss of function. When placed into this context, it may be better understood why joint disorders outrank all other musculoskeletal conditions in the horse with a reported $145 million spent in the diagnosis and treatment of osteoarthritis alone (Anonymous, 2000).

Joints may be divided into several categories according to composition and function. The three main types of joints include fibrous joints such as found within the axial skeleton, cartilaginous joints which compose the pelvic symphysis and articular joints of the appendicular skeleton which are of primary interest regarding lameness disorders in the horse (Dyce, 2010; Mcilwraith, 2016; van Weeren and Brama, 2001). Articular joints are further classified as diarthrodial, which allow for maximal motion limited only by periarticular structures. This joint structure is comprised of two congruent bone ends with a cartilage layer covering each of the opposing surfaces. A generally thin,

highly innervated and vascular subchondral bone plate provides underlying support for the articular cartilage. The articular cartilage is comprised of 1-12% chondrocytes, which contribute the extracellular matrix, and is without vascular, lymphatic, or neural supply (van Weeren and Brama, 2001). Periarticular structures aid in stability and include the joint capsule and/or soft tissue tendons, ligaments and muscles. The capsule itself is multifunctional with an outer fibrous layer that contributes to joint stability and is highly vascularized and a thin (1-3 cell layers thick) inner synovial membrane layer that regulates the composition of the synovial environment both by passive diffusion and through active secretion by synoviocytes (Mcilwraith, 2016). Synoviocytes are further classified as type A, which are responsible for phagocytosis, and type B, known for their secretory role in maintaining synovial fluid components, and type C which is considered a transitional cell type that may become either type A or type B (van Weeren and Brama, 2001). All cells contribute to maintaining joint homeostasis and are involved in the inflammatory processes resulting from joint disease and/or injury. The highly innervated joint capsule includes 4 types of receptors which are primarily responsible for pain associated with joint disease caused by trauma and inflammation due to lack of innervation within the articular cartilage itself (Mcilwraith, 2016). These receptors consist of low threshold Type 1 receptors located within the fibrous joint capsule, Type 2 which are located nearer the joint space and are activated only during movement of the joint, high threshold Type 3 located near insertion sites of periarticular soft tissues to bone, and type 4 which are located throughout the entire capsule and respond to both mechanical and chemical stimuli. Other contributors to pain perception include sensory innervation within the subchondral bone, marginal periosteum, and periarticular ligamentous attachments. Contained within the joint itself and bathing the articular

surface is synovial fluid with a molecular composition that closely resembles that of plasma only with a much higher hyaluronic acid concentration contributing to the viscosity of the joint fluid (Mcilwraith, 2016). This viscous nature of synovial fluid aids in creating a smooth gliding surface in addition to providing vital nutrients to the avascular articular cartilage and the ligaments found intraarticularly.

Cartilage Structure and Function

Ultimately, the ability of the joint to adeptly fulfill its requirements biomechanically are resultant of the characteristics of the articular cartilage (Mcilwraith, 2016). As a greater understanding of the complex molecular events which occur in order to create and maintain the structural environment of the joint surface, it is evident that several static and dynamic factors interplay to create a living, functional unit. As stated above, limited numbers of highly differentiated chondrocytes contribute to and maintain the extracellular matrix. Chondrocytes may be classified as differentiated fibroblasts from mesenchymal origin (Goldring et al., 2017; Mcilwraith, 2016). The extracellular matrix structure is comprised of water, collagen, and proteoglycans. The water content of cartilage is approximately 70-80% (van Weeren and Brama, 2001). Collagen makes up approximately 50% of the total dry weight. Articular cartilage is predominately composed of collagen type II as opposed to other body tissues (such as tendon, bone, and skin) where collagen type I is found in abundance. Both collagen types stem from the same tropocollagen molecule forming a continuous triple helix with its three large chains of amino acids. In collagen type II these are three α 1-chains that consist of repeating triplets of sequencing amino acids Gly-X-Y (X and Y can be any amino acid) (Todhunter et al., 1994). Collagen type I consists of two α1-chains and one α2-chain.

While collagen contributes tensile strength to the articular structure, proteoglycan aggregates are a major contributor to the compressive stiffness and biomechanically are responsible for the "creep" behavior exhibited by the cartilage in response to compressive forces (Todhunter et al., 1994).

There are four transitioning layers of cartilage which have been previously described. These layers include the superficial zone, the intermediate zone, the deep zone (all three of which form the hyaline cartilage) and the calcified layer nestled in between the more flexible hyaline cartilage and the rigid structure of the subchondral bone (Mcilwraith, 2016). The "tidemark" is a histologic term used to describe the distinct transition from the deep cartilage layer into the calcified layer (van Weeren and Brama, 2001). Hallmark characteristics of the superficial layer are flattened chondrocytes, densely packed collagen fibrils oriented parallel to the articular surface, a very high content of water, and limited number of proteoglycans (Burr and Allen, 2014). The middle zone differs in that this layer has a lower content of water, an increased number of proteoglycans, and reduced density of collagen type II fibrils. The middle zone is further characterized by more rounded chondrocytes arranged in a more haphazard orientation throughout the extracellular matrix. The deep zone is described as having the lowest collagen content and percentage of water, but the highest concentration of proteoglycans. Here, we begin to see chondrocytes arranged in distinct columns perpendicular to the calcified bone. The cells in this region are much more rounded and are most metabolically active (Burr and Allen, 2014). Although it has been long suggested that only one cell type, the chondrocyte, is present within articular cartilage there is evidence to suggest that articular cartilage progenitor cells exist in minute quantities (Mcilwraith, 2016).

Biomechanically, the interwoven proteoglycan aggregates and the collagen network provide a source of resilient strength analogous to that of a sponge (van Weeren and Brama, 2001). In the unloaded position, the negative charges of the sulfate groups within the proteoglycans attract water resulting in expansion of the extracellular matrix. This expansion is limited by the attachments of the proteoglycans to the collagen fibril mesh and a state of equilibrium is reached. When a load is applied, water is expressed from the extracellular matrix until a new equilibrium point is achieved. Once the load is removed, the process begins again with an equilibrial state being the constant goal. This is occurring during locomotion and results in a continuous ebb and flow of interstitial fluid to provide nutrition and to allow for removal of waste products, a process referred to as assisted diffusion. Any disruption within the collagen network or proteoglycan aggregates will result in detrimental effects to the articular unit (Boskey and Robey, 2013; Hoogen et al., 1999; Majd et al., 2014).

Despite the appearance of a homogenous tissue throughout the joint, it has been shown that areas under greater load adapt in that they develop a higher collagen and proteoglycan content than a region which does not experience constant loading (Brama et al., 2002). The variations in magnitude and type of load across the articular surface result in different biomechanical properties throughout the cartilage in response to the load to which it is exposed. These responsive changes within the cartilage to loading are determined during the first year of life in the horse with the most significant adaptive changes occurring within the first 5 months following birth (van Weeren, 2016; van Weeren and Brama, 2001). This further testifies to the adaptable and dynamic nature of cartilage during development.

Endochondral Ossification

The process of endochondral ossification is initiated with a hyaline cartilage template that is gradually transformed to mineralized bone through complex and coordinated activity conducted by chondrocytes, osteoblasts, and osteoclasts (Burr and Allen, 2014). Endochondral ossification may occur during longitudinal bone growth, within the articular epiphyseal complex, and in fracture healing (Mcilwraith, 2016). The process is initiated by the congregation of mesenchymal cells which then differentiate into chondroblasts in response to expression of the transcription factor, SOX-9 (Burr and Allen, 2014). Newly formed chondroblasts create an extracellular matrix that later envelops chondroblasts, transforming them into chondrocytes. A perichondrium surrounds the hyaline cartilage template and is responsible for the regulation of precursor cells involved in cartilage growth. More specifically, driven by RUNX2 the perichondrial cells differentiate into osteoblasts whose primary responsibility is to then form a collar of bone surrounding the diaphysis, after which the perichondrium transitions into the periosteum which houses osteogenic precursor cells. The newly formed bone collar prevents diffusion of nutrients, ultimately resulting in chondrocyte apoptosis and calcification of matrix. In response and with the aid of osteoclasts, vessels penetrate the bone collar and in the process a marrow cavity is created along with a primary ossification center. Similarly, secondary ossification centers are formed at the epiphyses of long bones. Cartilage canals in this region allow for vessels to penetrate within the deep cartilage layers (Burr and Allen, 2014).

With the expansion of the primary ossification center, the template develops a central diaphyseal region, including the marrow cavity, with two cartilaginous ends (Boskey and Robey, 2013; Burr and Allen, 2014; Semevolos, 2017). The epiphyseal plate

develops at the junction in between the marrow and the cartilage at either end of the template. This growth plate is primarily responsible for longitudinal bone growth. Endochondral ossification also occurs within the articular epiphyseal cartilage which is responsible for the reduced thickness of cartilage as the foal grows and for the increase in size of the joint surface during the process.

Within the epiphyseal growth plate and articular epiphyseal cartilage are 5 morphologically diverse zones (Burr and Allen, 2014). Each zone is classified by the cellular process that occurs during the process of endochondral ossification. The zone nearest the ends of the hyaline cartilage template is referred to as the resting zone. As this term may imply that cells in this region are inactive (nonproliferative), the term reserve zone may also be utilized interchangeably with resting zone. Within the resting reserve zone, hyaline cartilage matrix surrounds round, metabolically quiet chondrocytes. These resting chondrocytes are responsible for the secretion of parathyroid hormone-related protein (PTH-rP) resulting in delayed differentiation of neighboring chondrocytes and form a negative feedback loop with Indian Hedgehog (Ihh) pathway (Semevolos et al., 2002).

Active chondrocyte mitosis occurs within the proliferative zone (Burr and Allen, 2014). Chondrocytes in this zone develop a compressed appearance and begin forming a distinct stacked appearance. Several regulatory factors begin to play a role such Ihh, somatotropin/growth hormone, bone morphogenic proteins (BMPs), insulin-like growth factors, and the Wnt-ß-catenin pathway in order to encourage proliferation of chondrocytes. Fibrolast growth factor has been shown to be inhibitory to chondrocyte proliferation within this zone.

The hypertophic zone begins with a pre-hypertrophic phase followed by hypertrophy and apoptosis of chondrocytes in the lower portion of this zone (Burr and Allen, 2014; Semevolos, 2017). The primary promoter of chondrocyte hypertrophy is suggested to be thyroxine, whereas Ihh and PTH-rP are inhibitory. Within this zone, cells rapidly increase in size and production of extracellular matrix. In addition to the expected collagen type II, collagen type X makes an appearance which increases stiffness due to the presence of fibrils, but that concurrently reduces cell access to nutrition in this region (Boskey and Robey, 2013). Collagen type X is directly associated with vascular invasion. In the presence of cell hypertrophy, the abundant and compact matrix begins to calcify, transitioning into the fourth zone named the calcified cartilage zone. If cell death has not already occurred, apoptosis of chondrocytes ensues, likely as a factor of hypoxia (Burr and Allen, 2014). Chondrocytes continue to orchestrate calcification through the release of vesicles which contain components such as alkaline phosphatase that result in the aggregation of calcium and phosphorous and mineralization of the surrounding matrix. Vascular invasion continues in response to increased calcification and chondroclasts are recruited to the site specifically for the removal of cartilage. These chondroclasts make way for the fifth and final zone, the zone of ossification, in order that new woven bone may be produced by incoming osteoblasts. The presence of osteoclasts results in the remodeling of woven bone into lamellar bone. Longitudinal growth of bone will continue to occur until the epiphyseal growth plate becomes entirely calcified, noted as closure of the growth plate (Semevolos, 2017; 2014).

Osteochondrosis

The term *osteochondrosis* is widely used to describe a disruption within the process of endochondral ossification during longitudinal bone growth of the diaphysis and of the epiphysis in mammalian bone (Denoix et al., 2013). The disorder was first described in horses 1947 and became clinically important in the 1970's (Mcilwraith, 2016). It has been previously reported on Northwestern Europe that up to 20-25% of the equine population are born each year with one or more forms of osteochondrosis (van Weeren and Barneveld, 1999). Several predilection sites have been identified in the horse and include (in order of prevalence) the distal intermediate ridge of the tibia, lateral trochlear ridge of the talus, medial malleolus of the tibia, medial trochlear ridge of the talus, dorsal distal mid-sagittal ridge of the cannon bone, dorsal margin of the first phalanx, the lateral trochlear ridge of the femur, medial trochlear ridge of the femur, distal patella and intertrochlear groove of the femur. The disease may occur unilaterally or bilaterally with up to 20% of stifle osteochondrosis and up to 10% of hock lesions occurring in both joints (van Weeren, 2016). These lesions manifest clinically as defects within the articular cartilage, with or without involvement of subchondral bone. Frequently, diagnosis is made with the use of available imaging techniques (radiography, ultrasound, magnetic resonance imaging, computed tomography) after onset of effects secondary to inflammation such as joint effusion with or without clinical lameness. These symptoms are representative of advanced stages of the disease process.

In horses, further definitions have been implemented to characterize the dynamic process and the variable clinical and histopathologic findings of the disease (Denoix et al., 2013). *Osteochondrosis latens* has been used to describe early lesions with lack of evidence of reactive changes within the cartilage. The term *osteochondrosis manifesta*

refers to the ability of early lesions to either spontaneously regress or succumb to environmental factors which might lead to progression towards the clinical manifestation of the disease known as *osteochondritis dissecans* in which there is evidence of articular cartilage defects and/or osteochondral fragmentation. Once considered a part of the developmental orthopedic diseases, a recent study proposed the use of the term *Juvenile Osteochondral Conditions* in reference to disorders occurring during critical periods of longitudinal bone growth. This definition excludes disorders such as angular limb deformities, physitis, and vertebral stenotic myelopathy (Denoix et al., 2013).

The pathophysiology of osteochondrosis is poorly understood. The bulk of studies within the last 20 years have investigated the multifactorial etiology of osteochondritis dissecans, identifying many genetic and nutritional factors (reviewed in Semevolos, 2017). However, a recent shift in focus has turned towards the understanding of the early pathogenesis of osteochondrosis within the first 6 months of life. During this critical period of growth, several biomechanically weakened areas have been identified within the epiphyseal articular cartilage complex, including the effects of shearing forces along the osteochondral junction and the failure of cartilage canals (Laverty and Girard, 2013; Olstad et al., 2013; Pool, 1993). However, much knowledge is to be gained in order to obtain an understanding of the early pathogenesis of the disease.

Genes of Interest

To the authors knowledge, the current study is the most comprehensive targeted gene study of its kind. We have evaluated 18 genes, the majority of which have yet to be evaluated for their role in early equine osteochondrosis (Table 1). By evaluating gene and protein expression, we are able to fill in some of the gaps in our current knowledge of the

complex cellular interactions in both endochondral ossification and in early osteochondrosis.

Aggrecan is the primary proteoglycan found in articular cartilage that has both structural and developmental properties (Kiani et al., 2002). Aggrecan is comprised of chondroitin sulphate and keratin sulphate joined to a core protein with 5 known domains. Three of these domains are globular and are described as G1, G2, G3. Aggregation occurs through the binding of hyaluronan at the N-terminus, G1. This domain resembles a “link protein”, known for stabilizing this connection. It is this aggregation that contributes to the biomechanical properties of the cartilage by forming structurally sound hydrogel complexes throughout the extracellular matrix. The expression of aggrecan by chondrocytes is critical to interactions between cells and with the surrounding matrix. The crucial role of aggrecan in early skeletal development has been identified in a study in which a functional null mutation for the gene was evaluated in mice (Boskey and Robey, 2013). These mice suffered from severe dyschondroplasia and thus confirmed the role of aggrecan in the endochondral ossification process. Aggrecan is found mainly within cartilage with much lower concentrations in bone, where its role is largely unknown; it is speculated that the effects of aggrecan in bone may be related to mineralization of the growth plate (Kiani et al., 2002).

Aggrecan is cleaved at five distinct sites on the core protein by aggrecanases. Aggrecanase-1 is a metalloprotease belonging to the ADAMTS family and may also be referred to as ADAMTS-4. It has been extensively evaluated in many human osteo and rheumatoid arthritis studies where it has been shown to be secreted by synoviocytes in response to inflammatory cytokines, in particular TGF-β (reviewed in Firestein, 2017). Aggrecanase-1 has also been shown to degrade cartilage oligomeric matrix protein

(COMP), in addition to Aggrecan. Aggrecan contains two proteolytic sites, one for aggrecanase and the other for matrix metalloproteases (MMPs). MMP 1 and 3 are also known as Collagenase 1 and stromelysin-1, respectively (Goldring et al., 2017). MMPs are secreted by chondrocytes in inactive forms until a stimulus, such as an inflammatory cascade, is initiated. For example, cartilage degradation may occur from the activation of plasmin, which then activates MMP-3, which in turn activates collagenases.

Transforming growth factor beta 3 (TGF-β3) abounds in skeletal bone, the majority of which is to be found in its inactivated form (patterning factor) (Thorp et al., 1992). A study performed in three-week-old chicks suggested the role of TGF-β in mineralization of the extracellular matrix based on its expression in hypertrophic chondrocytes and osteoblasts (Thorp et al., 1992). This study also suggested a role of TGF-β in synthesis of matrix due to its presence in transitional chondrocytes, resulting in an increased expression of collagen type II mRNA. TGF-β may also play a part in the process of resorption and construction of new bone as concentrations are found within osteoclasts and chondroclasts during endochondral ossification. In a study evaluating growth factor expression in osteochondrosis affected pigs, it was observed that TGF-β was significantly reduced within chondrocytes associated with osteochondrosis lesions (Thorp et al., 1995). This study suggested a similar process to that described in avian dyschondroplasia.

Cartilage oligomeric matrix protein (COMP), is a large pentameric glycoprotein that may be found in tendon and cartilage. Three main roles for COMP have been described and include binding of extracellular matrix proteins, facilitating polymerization of type II collagen fibrils, and regulation of chondrocyte proliferation (Posey and Hecht, 2008). Studies in humans have evaluated several disorders such as pseudoachondroplasia

and multiple epiphyseal dysplasia which occur in response to mutations within the COMP gene (Posey et al., 2018). These studies are in agreement of the mechanism that retained COMP within the endoplasmic reticulum causes oxidative stress and resultant chondrocyte death (Hecht et al., 2004; Posey et al., 2018; Schmitz et al., 2008).

The SOX subfamily is a group of complex proteins with a characteristic high-mobility-group DNA-binding domain (Lefebvre et al., 2001). Its role in transcription activation is of great interest and has led to several studies evaluating the potential influence it may have on chondrocyte differentiation. A comprehensive review evaluated the expression of SOX 5, 6, and 9 during chondrocyte differentiation (Ikeda et al., 2005). In the early phases of endochondral ossification, mesenchymal conglomerations form and receive signals that direct their differentiation into specific cell lineages. This study showed the importance of SOX 5 and 6 after these mesenchymal condensations are formed, while SOX 9 was shown as important before and after condensation. All three are required for chondrocyte differentiation to occur appropriately.

Basic fibroblast growth factor (bFGF) is classified as a “patterning factor”, which means that it plays a role in determining the actual shape and size along with formation and shape of specific skeletal components (Lefebvre et al., 2001). bFGF is a mitogen and has been shown to stimulate the mitotic activity of growth plate chondrocytes and may inhibit their terminal differentiation (Nagai et al., 1995). Due to its bioactive form and expression in osteoblasts, it is thought to be a local regulator of bone formation.

PRG4 (Lubricin) is a mucinous glycoprotein secreted by chondrocytes located within the superficial zone and by fibroblasts within the synovium (Majd et al., 2014; Waller et al., 2013). It is referred to as a boundary lubricant with the primary role of creating a frictionless gliding surface to preserve the integrity of the articular cartilage

surface. Collagen type II and hyaluronan have been shown to essentially trap lubricin into the superficial cartilage layer in order to perform this duty (Majd et al., 2014). Lubricin has been shown to be important in maintaining the structural integrity of superficial layers of cartilage, has shown to be protective of chondrocytes, and reduces the coefficient of friction (Waller et al., 2013). In one study using rats with experimentally induced post-traumatic osteoarthritis, intraarticular lubricin injections were shown to have beneficial effects of reducing cartilage damage (Teeple et al., 2011).

Jagged-2 is a ligand that interacts with the Notch signaling pathway, which has been shown to be of critical importance during early embryonic development and in chondrocyte differentiation (Hayes et al., 2003). This study reports the presence of Jagged -2 in chondrocytes throughout each of the layers of the developing articular cartilage of mice, with higher concentrations of Jagged 2 expressed at the surface of the developing joint. This expression may be important in determining cell fate in this important signaling region. Jagged 2 was also noted within maturing chondrocytes of the growth plate, suggesting the role of Jagged 2 interactions with the Notch signaling pathway as important for both joint development and elongation of long bones. This interaction has proven very complex as the Notch signaling pathway has been shown to be both inhibitory in early stages and stimulatory in the later stages of chondrocyte differentiation, with all activating ligands incompletely identified (Hosaka et al., 2013).

Ephrin B2 is a ligand that attaches to ephrin receptors of the tyrosine kinase family. Parathyroid hormone (PTH) stimulates release of Ephrin B2 from osteoblasts and amplifies new bone formation (Matsuo and Otaki, 2012). Ephrin B2 also plays a role in remodeling of bone.

Runx2 is a critical transcription factor for osteoblast differentiation (Komori, 2015). Runx2 has also been shown to be a major signal for endochondral ossification and chondrocyte maturation. In one study evaluating the role of Runx2 in both chondrocyte differentiation and in new bone formation in chick embryos and mice, the multifactorial role of the gene was revealed. It was shown that Runx2 played a role in chondrocyte differentiation, vascular invasion, osteoclast/osteoblast differentiation, and periosteal bone formation (Komori, 2018). More specifically, Runx2 is a primary regulator of Ihh expression and promotes proliferation of chondrocytes (Komori, 2015).

Gremlin 1, a glycoprotein, is considered a BMP antagonist (Gazzerro et al., 2007). BMP antagonists help to create a tightly regulated system in which proper formation of the limbs occurs. Specifically, Gremlin 1 null mice were shown to have increased bone formation and osteoblastic activity (Gazzerro et al., 2007). Without their countering effects of Gremlin 1, severe limb deformations occur during embryogenesis, and renal and lung dysfunction cause death in utero. Upregulation of this gene has been linked to osteopenia due to reduced numbers of osteoblasts (Gazzerro et al., 2005).

Osteocalcin is a small non-collagenous protein highly expressed in bone and in calcified cartilage (Boskey and Robey, 2013). Osteocalcin has a very high affinity for hydroxyapatite and is involved in hydroxyapatite crystal structure formation. In addition to structural functions, osteocalcin sets the stage for bone resorption by signaling recruitment for osteoblast and osteoclast precursor cells (Hall, 2015). It has been shown to have an important role in coordinating chondrocyte calcification.

Collagen is what is called a “trimeric” molecule which consists of α-chain subunits that form a strong triple helix structure (Boskey and Robey, 2013). These α-chains may be all the same or can vary in structure. Collagen may be divided into 29

known types, with Collagen type I being found predominantly in bone and comprising approximately 90% of its structural proteins, whereas collagen type II dominates the articular cartilage structure (Mcilwraith, 2016). Both types are classified as “fibrillar” collagens, indicating that fully formed collagen molecules are initially formed by proteolysis of a precursor molecule (tropocollagen, procollagen). Collagen type X is considered to be “non-fibrillar”, characterized by triple helical structures that may either be shorter or longer than those found in fibrillar type collagen. Collagen type X is found mainly within calcified cartilage, and most notably is found within the growth plate. Although collagen may not be directly related to the orchestration of calcification, it has been shown to provide a scaffold that aids in organization of the newly deposited mineral in bone.

Several recent studies have evaluated the potential molecular events that result in abnormal extracellular matrix composition allowing for a disruption in ossification of the cartilaginous template (Laverty and Girard, 2013; Lecocq et al., 2008; van de Lest et al., 2004). As described above, extracellular matrix plays a pivotal role in tensile and compressive strength of cartilage. Weakened matrix near the osteochondral junction is more likely to be affected by shear forces resulting in osteochondral separation and osteochondrosis lesions. Previous studies have identified abnormal type II collagen production in osteochondrosis lesions (Laverty et al., 2002), but cell-specific information on matrix regulation has not been performed.

Table 1. Our targeted study evaluated 18 genes with known roles in bone/cartilage regulation.

Gene	Classification	Significance
Collagen Type I	Structural protein	90% of bone matrix; Scaffold that binds other proteins to encourage deposition of hydroxyapatite
Collagen Type IIb	Structural protein	Predominates in articular cartilage
Collagen Type X	Structural protein	Found in hypertrophic cartilage
Aggrecan	Significant proteoglycan of articular cartilage	Contributes to load bearing properties by forming hydrogel with hyaluronan
Aggrecanase-1	Metalloprotease belonging to the ADAMTS family and may also be referred to as ADAMTS-4	Degradation of aggrecan
MMP-1	Matrix metalloproteinase; fibroblast collagenase	Matrix degradation, Col types I-III
MMP-3	Matrix metalloproteinase; Stromelysin 1	Matrix degradation
SOX 5	Transcription factor	Involved in embryonic development of cartilage; Activates transcription of Col2a and AGC1
SOX9	Transcription factor	Critical for proper embryonic development of cartilage
Jagged 2	Ligand that interacts with the Notch signaling pathway	Critical importance during early embryonic development and in chondrocyte differentiation
COMP	Glycoprotein found in tendon and cartilage	Binds extracellular matrix proteins, facilitating polymerization of type II collagen fibrils, and regulation of chondrocyte proliferation
Runx2	Transcription factor	Osteoblast differentiation
Gremlin 1	Bone morphogenic protein antagonist	Embryonic development
Ephrin B2	Ligand	PTH stimulates release of Ephrin B2 from osteoblasts and amplifies osteoblastic new bone formation
Lubricin/PRG4	Mucinous glycoprotein	Secreted by chondrocytes located within the superficial zone and by fibroblasts within the synovium
Osteocalcin	Non-collagenous protein	Role in mineralization
bFGF	Mitogen	Stimulates mitotic activity of growth plate chondrocytes, may inhibit terminal differentiation
TGF-ß3	Transcription factor	Role in resorption and construction of new bone

Chapter 2: Literature Review

The majority of osteochondrosis research over the last two decades has been focused on the multifactorial etiology of the disease and surrounding treatment options for end-stage disease. More recently, a shift in focus has occurred with researchers honing in on the complex molecular events involved in the pathogenesis of early osteochondrosis. Research efforts in this area have produced several hypotheses as to the inciting cause of this disorder including alterations in cellular mechanisms involved in chondrocyte differentiation and extracellular matrix composition, failure of cartilage canals resulting in focal areas of osteonecrosis, and shearing forces at biomechanically inferior locations along the osteochondral junction (following trauma) (Carlson et al., 1995; Laverty et al., 2002; Lecocq et al., 2008; Mirams et al., 2009; Olstad et al., 2013; Pool, 1993; Riddick et al., 2012; Semevolos et al., 2002). This specialized area of research can certainly prove challenging as the processes of endochondral ossification and chondrocyte differentiation involve a multitude of complex and overlapping pathways that have yet to be entirely identified. The dynamic, ever changing quality of these molecular events leads to challenges in determining when to coordinate sample collection and in the interpretation of results. Each study contributes a small piece of the puzzle that slowly unfolds a larger picture. This picture will eventually open the doors to future therapeutic and diagnostic tools to aid in the early identification and prevention of the disease across multiple species, if a common pathogenesis is identified.

Chondrocyte Differentiation

The transformation of the cartilage template within the epiphysis occurs in a well-regulated, step-wise fashion. Key molecular events that must occur include proliferation

and differentiation of chondrocytes, production of expansive extracellular matrix, followed by invasion of vascular supply and mineralization of the extracellular matrix. The earliest appearance of osteochondrosis has been previously described as a small island of retained cartilage which juts into the subchondral bone (Olsson, 1987). Characteristic of these retained cartilage cores are congregations of what appear to be small, rounded pre-hypertophic chondrocytes (Jeffcott and Henson, 1998). It has been suggested that this disruption within the normal endochondral ossification process, which prevents the following stages of formation of vascular supply and mineralization of the matrix, leads to thickened cartilage and cartilage separations associated with clinical osteochondrosis dissecans (Ekman and Carlson, 1998).

Due to the complex nature, chondrocyte differentiation has not yet been defined in its entirety. However, a number of studies have explored some of the known pathways in order to determine their relevance to the pathogenesis of early osteochondrosis including parathyroid hormone-related protein, Indian Hedgehog, and bone morphogenic proteins (Duesterdieck-Zellmer et al., 2015; Semevolos et al., 2002, 2005, 2006). The novel findings of Semevolos *et al*. defined an age-related variance in the expression of these factors during early growth phases in horses. In one study, an overall activation of the bone morphogenic protein family was found in cartilage maturation, in addition to a reduction in PTH-rP and no significant changes in Ihh expression. These findings laid the groundwork for future studies to determine variations in diseased articular cartilage (Semevolos et al., 2006). Follow up studies further explored known pathways involved in chondrocyte proliferation and differentiation.

Another study revealed an increased expression of Ihh in the deeper layers of osteochondrosis-affected cartilage (Semevolos et al., 2005). This was significant as Ihh is

generally found at minimal concentrations within normal articular cartilage. However, expected changes of an increased expression of the transcription factor Gli1 and its ability to alter hedgehog expression were not observed. These unexpected results led to the hypothesis that an unknown mechanism is creating delayed endochondral ossification due to deviation from appropriate chondrocyte differentiation signaling mechanisms. A study conducted by Duesterdieck-Zellmer *et al*. (2015) further explored the dynamic nature of the endochondral ossification process by evaluating the presence of known pathways within a particular age range among growing foals. Using laser capture microdissection, chondrocytes along the osteochondral junction and surrounding cartilage canals were specifically evaluated in both pre-adolescent and neonatal foals. Results of this study described a trend for higher gene expression of PTHrP along the cartilage canals and Ihh along the osteochondral junction during the early growth period. Differential expression of Wnt signaling pathways and their inhibitors were also described and used as evidence for the complex molecular interactions occurring during early articular-epiphyseal cartilage growth.

The Wnt signaling pathway is known for its role in chondrocyte differentiation and maturation in early articular cartilage development (Duesterdieck-Zellmer et al., 2015). The Wnt/ß-catenin and retinoic acid pathways have been shown to influence the roles of chondrocytes and turnover of the extracellular matrix. Kinsley *et al*. (2015) further demonstrated that alterations within the Wnt signaling pathway in chondrocytes located near cartilage canals may be the primary factor for dysregulation of chondrocyte maturation in early osteochondrosis.

Although the final fate of mature chondrocytes has been much debated, one study explored the presence of apoptotic markers along the osteochondral junction in an effort

to correlate aberrant cell death to the presence of osteochondrosis (Semevolos et al., 2018). This study concluded that due to the presence of several apoptotic markers in osteochondrosis affected samples, uncoordinated cell death was likely occurring either in direct or indirect relation to the disease.

Despite several hypotheses that osteochondrosis is a result of failure of chondrocytes to undergo hypertrophy, a study by Mirams *et al*. (2009) indicated that this may not be the case. In this study, osteochondrosis was induced by feeding a high energy diet to young, growing foals for a period of 8-15 weeks. Resultant OC lesions were evaluated by PCR and histologically in order to determine presence of known regulators for chondrocyte differentiation throughout the endochondral ossification process. Increased expression of MMP-13, collagen type I, and Runx2 within the lesions indicated that hypertrophic chondrocytes were certainly present.

Extracellular Matrix

The extracellular matrix also plays an important role in the pathophysiology of osteochondrosis. During endochondral ossification, the original matrix is replaced with a composite more adeptly suited for the biomechanical demands of the newly formed bone, in which the role of collagen has been shown to be critical in the processes of both remodeling and calcification (van de Lest et al., 2004). According to one study, age and exercise directly influence the heterogeneity of articular cartilage, with the most critical changes in collagen composition occurring within the first 5 months after birth (Brama et al., 2002). Lecocoq *et al*. (2008) suggested that distinct differences in collagen type II structure within osteochondrosis-affected cartilage could cause weakening of the extracellular matrix and be a predisposing factor for disease. In an attempt to define the

inciting molecular events involved in early equine osteochondrosis, Riddick *et al*. (2012) discovered an upregulation of genes associated with matrix degradation products in osteochondrosis-affected foals less than 6 months of age. Namely, matrix metalloproteinase-13 and matrix metalloproteinase-3, involved in the degradation of collagen type II and aggrecan, respectively, were shown to have increased gene expression in osteochondrosis. Both may have a role in the degradation and resultant weakening of the extracellular matrix. Other described changes within the extracellular matrix of osteochondrosis affected cartilage include a reduction in chondroitin sulfate and proteoglycan production (Hoogen et al., 1999). Chondroitin sulfate is known for resisting compression and adding to the structural integrity to cartilage, while aggrecan provides structural compressive properties and aids in water retention (Laverty and Girard, 2013; Lillich et al., 1997). Laverty *et al*. (2002) demonstrated an increase in collagen type II cleavage by collagenases in osteochondrosis affected horses, with no concurrent increase in breakdown of proteoglycans. With evidence to suggest that weakened extracellular matrix occurs at predisposed osteochondrosis sites, the question remains whether these changes are inciting causes or secondary effects in the pathologic process (Figure 1).

Failure of Cartilage Canals

The role of cartilage canals in epiphyseal osteochondrosis has been extensively studied. As described in the previous chapter, the role of cartilage canals is vital in order to sustain and provide nourishment to the markedly thick epiphyseal cartilage (Figure 2). These cartilage canals are oriented both parallel and perpendicular to the ossification front. Surrounding chondrocytes are susceptible to focal areas of necrosis when anastomoses of vessels fail, resulting in failure of cartilage canals. As the foal grows,

these cartilage canals regress in a process called chondrification (Ytrehus et al., 2004). Cartilage canals have been found to be present in the epiphysis of equine fetuses at 130 days gestation and are normally absent by 6 months of age (Shingleton et al., 1997). Several hypotheses have formed surrounding the cartilage canals as a potential inciting cause for osteochondrosis due to focal areas of necrosis. In a study evaluating the distal femur of swine, Ytrehus *et al*. (2004) described the histologic findings of normal and abnormal chondrification. It was noted that a disruption in cartilage canal blood supply was evident where anastomoses of cartilage canal vasculature and that of bone marrow occurs. In this study it was suggested that microtrauma could be the primary cause of such findings which ultimately lead to osteochondrosis (Ytrehus et al., 2004). Carlson *et al*. (1995) earlier discovered these findings in horses showing a failure of cartilage canal blood supply as a significant factor associated with osteochondrosis lesions. In addition, the 2013 study by Olstad *et al.* (2013) described the importance of cartilage canals in foals that after an experimental disruption of blood supply to the distal femur, developed areas of focal chondronecrosis. These areas of chondronecrosis resulted in delayed endochondral ossification and lesions consistent with osteochondrosis (Olstad et al., 2008, 2011, 2013; Semevolos, 2017).

Biomechanical Forces

Predilection sites for osteochondrosis may be directly correlated to the biomechanical forces applied to the rapidly growing epiphyses (Brama et al., 2002). Several studies have identified risk factors related to exercise and housing arrangements in young, growing foals that prevent adequate or consistent loading of the joint and is directly associated with prevalence of osteochondrosis (Brama et al., 2002; Lepeule et al., 2013). Pool

(1986) introduced a theory that biomechanical forces, in particular, shearing forces along the osteochondral junction act as a primary inciting cause for osteochondrosis. In light of the previously discussed studies in which changes in collagen composition and orientation and the timeline during which anastomoses of vessels occur during early growth and loading of the joint, these factors and others may create biomechanically vulnerable areas that correspond with known sites of osteochondrosis.

Although evaluated separately, each of these factors may be closely intertwined. Our rationale for the current study, therefore, is that identification of differences in levels of gene and protein expression of matrix molecules and their regulators in cartilage cells surrounding cartilage canals and the cartilage-bone junction in early osteochondrosis and normal cartilage will help determine the role of extracellular matrix regulation in the pathogenesis of early equine osteochondrosis.

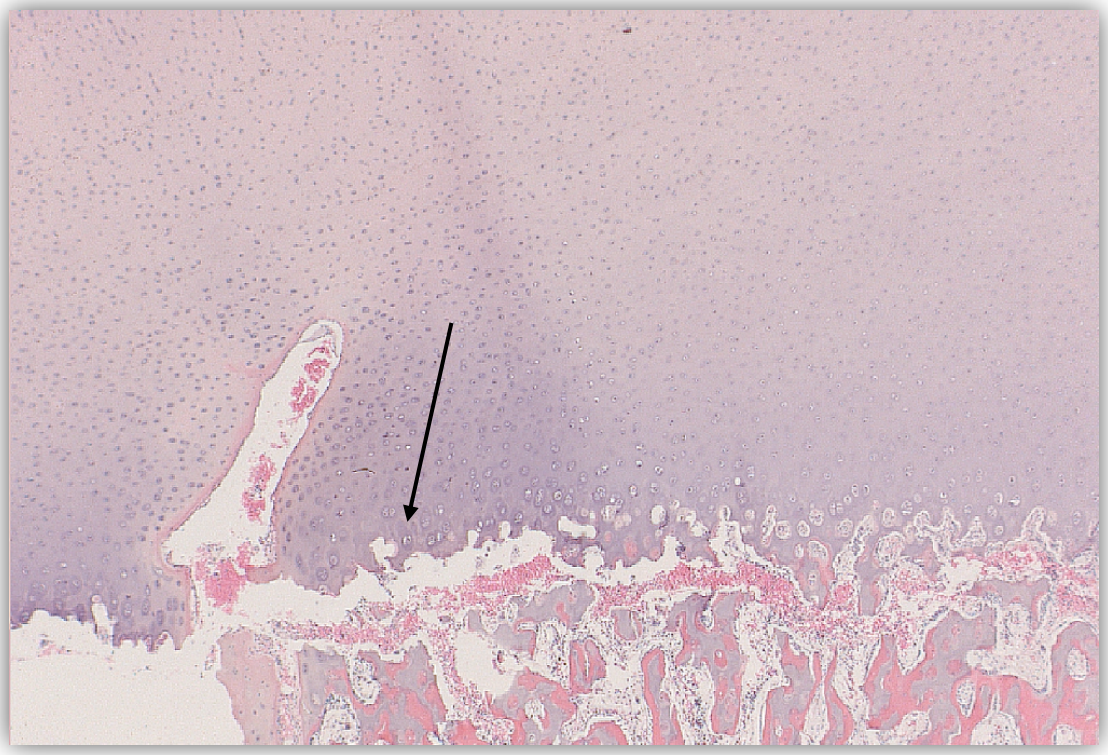

Figure 1. One hypothesis for the pathogenesis of osteochondrosis involves weakened extracellular matrix which results in failure at specific sites, such as shearing forces along the osteochondral junction resulting in separation as depicted by the black arrow in this photomicrograph.

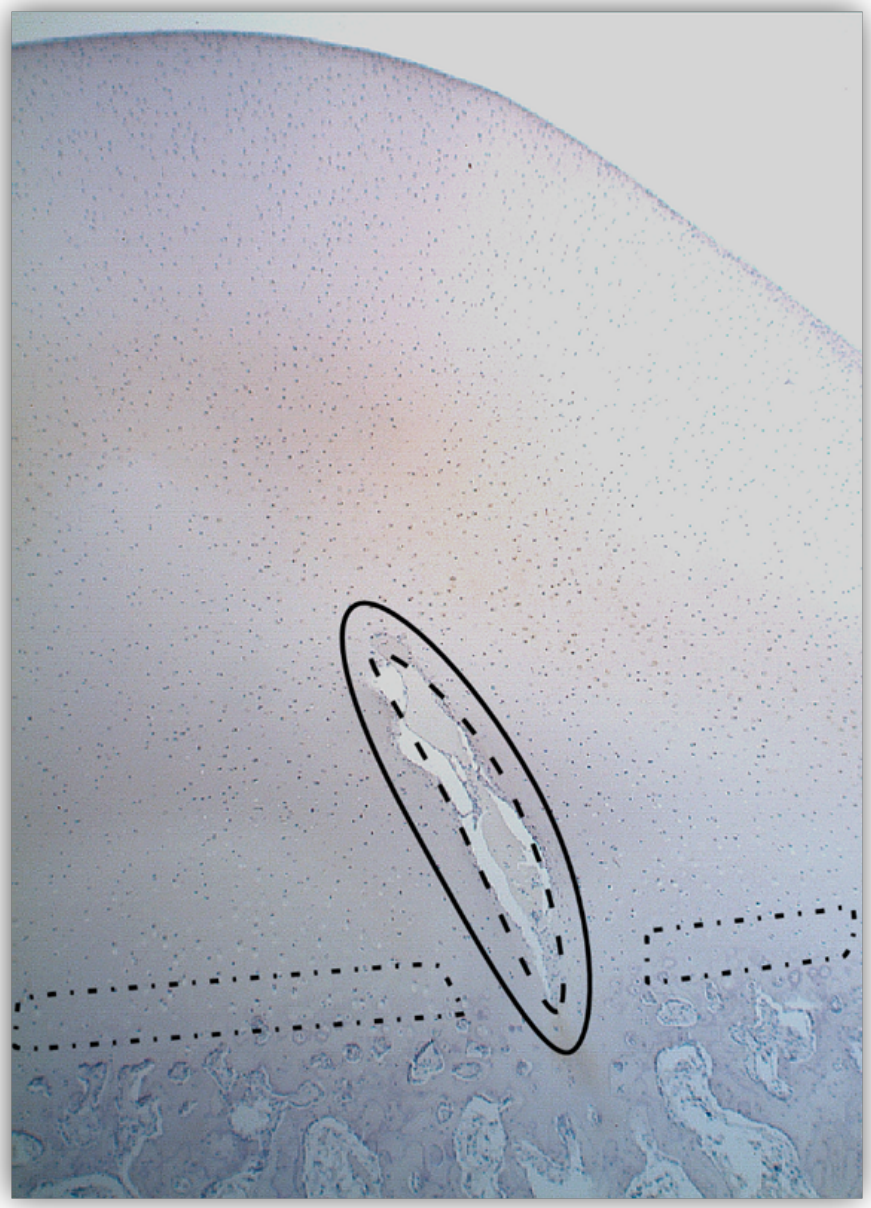

Figure 2. Photomicrograph depicting the cartilage canals (inner dashed and outer solid lines) and osteochondral junction (dashed-dotted lines), two sites of interest in the current study. Samples were harvested using laser capture microdissection in order to obtain cells specifically within these regions.

Chapter 3: Materials and Methods

Sample Collection

Archived paraffin-embedded osteochondral samples (6 osteochondrosis-affected, 8 normal controls) and cDNA from chondrocytes captured with laser capture microdissection (4 OC, 5 normal controls) from frozen sections were used in this project (Figure 3). These samples were previously obtained from the lateral trochlear ridges of femoropatellar joints in foals ranging from 1-6 months of age. Institutional Animal Care and Use Committee approval was obtained. Laser-captured samples included: 1) small chondrocytes surrounding cartilage canals near the osteochondral junction and 2) hypertrophic chondrocytes along the osteochondral junction.

Sample Evaluation and Classification

All osteochondral samples were evaluated grossly at the time of harvest and histologically following H&E staining in order to classify them, as previously described (Riddick et al., 2012). Briefly, 6 foals were determined to have early OC, and 8 were classified as normal. In OC samples, 5 foals had separation along the osteochondral junction and 3 foals had locally thickened cartilage (2 with concurrent osteochondral separation), all without concurrent superficial lesions (Table 2).

Real-time quantitative PCR

Following RNA isolation from laser-captured cells, reverse transcription was performed to create cDNA, using random hexamers. In order to maximize the amount of cDNA available from these limited samples, logarithmic pre-amplification with equine specific primers and the Taqman PreAmp Master Mix kit was performed. Equine specific primers and probes for the TaqMan® system were previously designed (Collagen type I,

Collagen type IIB, Collagen type X, Osteocalcin, SOX 5, SOX 9, TGF-β3, MMP-1, MMP-3, bFGF, COMP, Jagged2, Ephrin-β2, Gremlin1, PRG4, Runx2, Aggrecan, Aggrecanase), meeting specific criteria of Primer Express software version 2.0b8a (Applied Biosystems, Foster City, CA). Real-time quantitative PCR of diluted pre-amplified cDNA samples (1:20) was then performed according to standard protocol, using the ABI StepOnePlus real-time PCR system and software (Applied Biosystems, Foster City, CA) (Kinsley, 2015). Probes were labeled with a reporter dye, FAM (6-carboxy-fluroscein), and a quencher dye, TAMRA (6-carboxy-teramethylrhodamine). For each experimental sample the amount of target cDNA was determined by a relative standard curve, using the same calibrator for all experiments. PCR was performed in duplicate using 20µl 25 final reaction mixture of 2X Taqman® Gene Expression Master Mix, 250nM probe, 900nM forward and reverse primers, and 7.5µl pre-amplified sample cDNA and 18S RNA was used as the housekeeping gene for normalizing gene expression. After a two-minute incubation at 50ºC activating uracil-DNA glycosylase (UDG) and ten-minute incubation at 95ºC to deactivate UDG and activate AmpliTaq®Gold DNA polymerase, 40 PCR cycles of fifteen seconds of 95ºC followed by 1 minute of 60 ºC were run.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin-embedded osteochondral sections using mouse monoclonal α-human primary antibodies for Sox9, Collagen type IIB, Osteocalcin, and rabbit polyclonal α-human primary antibodies for Lubricin (Research Diagnostics, Inc., Flanders, NJ) and Supersensitive Link-label Multilink Immunohistochemistry System (Biogenex, San Ramon, CA) for Osteocalcin, Sox 9,

Lubricin, and Collagen type IIB. Negative procedural controls were confirmed by using non-immune serum in place of primary antibody. Following deparaffinization, osteochondral sections (6 OC, 8 normal) were either incubated at 37^{o}C for 60 minutes under a solution of testicular hyaluronidase (cartilage samples) or for 5 minutes under pepsin (connective tissue controls) to expose the antigen. Endogenous peroxidases were quenched with hydrogen peroxide and methanol. Non-immune goat serum was applied for 30 minutes (polyclonal primary antibodies only), and the primary antibody was applied for 60-90 minutes at room temperature. Secondary biotinylated multilink antibodies were applied, followed by labeling with streptavidin conjugated peroxidase, and then applying diaminobenzidine tetrachloride (DAB) as a chromogen for production of color product. The sections were counterstained with Harris hematoxylin and mounted for microscopy.

Immunohistochemistry scoring

Using the previously described HSCORE scoring system (Brun et al., 2012; Schatz et al., 2012) samples were evaluated by two blinded investigators. Scoring of each sample was determined for specific cell populations (chondrocytes surrounding the cartilage canals and chondrocytes adjacent to the osteochondral junction) by using the equation HSCORE=Σi i*Pi which calculates the sum of the percentage of positive staining cells or matrix (Pi) at each intensity multiplied by its respective intensity score (i). Staining intensity scores were from 0-3 as follows: 0=no staining, 1=mild staining, 2=moderate staining, and 3=strong staining. Scores were averaged at each location by both investigators (SAS, SKG). Superficial, middle, and deep cartilage layers were also scored, averaged, and the HSCORE calculated for each cartilage layer (Figure 4).

Statistical analysis

Quantitative comparisons from real-time PCR assays were compared for each site (cartilage canal, osteochondral junction) between OC and normal horses using a Wilcoxon rank sum test. *P*-values for all gene expression comparisons for each site were then ordered from smallest to largest and the significance level was corrected (q*) to control the false discovery rate, according to Benjamini and Hochberg (Benjamini and Hochberg, 1995). Using a Bonferroni correction and false discovery rate (FDR)<0.1, the corrected significance level for gene expression comparisons at the cartilage canal site was q*= 0.0055 and the osteochondral junction site was q*=0.0055. Gene comparisons were considered to be significant if *P*-values ≤ q* for the respective site. The term trend was used for *P*-values <0.05 prior to Bonferroni correction.

Immunohistochemistry scores were compared between OC and normal horses using a Wilcoxon rank sum test ($P<0.05$). Samples were placed into three groups for statistical analysis; normal or osteochondrosis-affected. Of those samples that had a paired contralateral limb, samples were averaged for a single total value to be assigned to each individual foal. Values from the averaged HSCOREs for the superficial, middle, deep, total average cartilage value, osteochondral junction, cartilage canals and bone were evaluated. Extracellular matrix was evaluated separately from bone and cartilage cells for each layer (Tables 3-8).

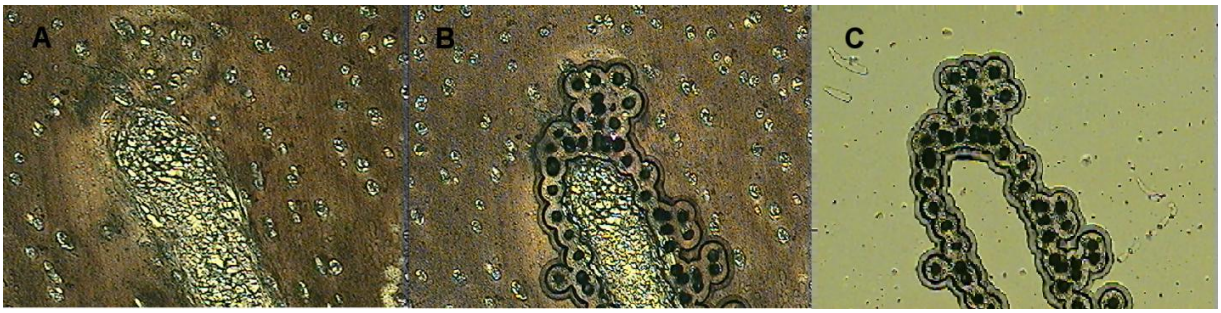

Figure 3. Photomicrograph depicting laser capture microdissection of chondrocytes surrounding the cartilage canal of a 4-month-old foal. A) Image of cartilage canal prelaser B) Following ignition of laser, cells of interest are captured C) Captured chondrocytes are harvested in the cap to be used for further analysis.

Table 2. Summary of horses and samples from the study with descriptions of the gross and histologic abnormalities.

Foal	Classification	Age (months)	Sex	Description
1	Osteochondrosis	5	Colt	Cartilage easily separated from subchondral bone, chondronecrosis along osteochondral junction
2	Osteochondrosis	4	Colt	Thickened cartilage easily separated from subchondral bone
3	Osteochondrosis	5	Colt	Thickened cartilage easily separated from subchondral bone
4	Osteochondrosis	4	Filly	Cartilage easily separated from subchondral bone, subchondral bone hemorrhage
5	Osteochondrosis	1	Filly	Thick cartilage with osteochondral junction containing fingerlike projections of cartilage
6	Osteochondrosis	5	Filly	Thin cartilage distal medial trochlear ridge, delayed ossification with extremely thickened cartilage proximally, cartilage easily separated from subchondral bone, chondronecrosis along osteochondral junction
7	Normal	4	Colt	No lesions
8	Normal	4	Filly	No lesions
9	Normal	4	Filly	No lesions
10	Normal	5	Filly	No lesions
11	Normal	4	Filly	No lesions
12	Normal	4	Filly	No lesions
13	Normal	6	Filly	No lesions
14	Normal	3	Filly	No lesions

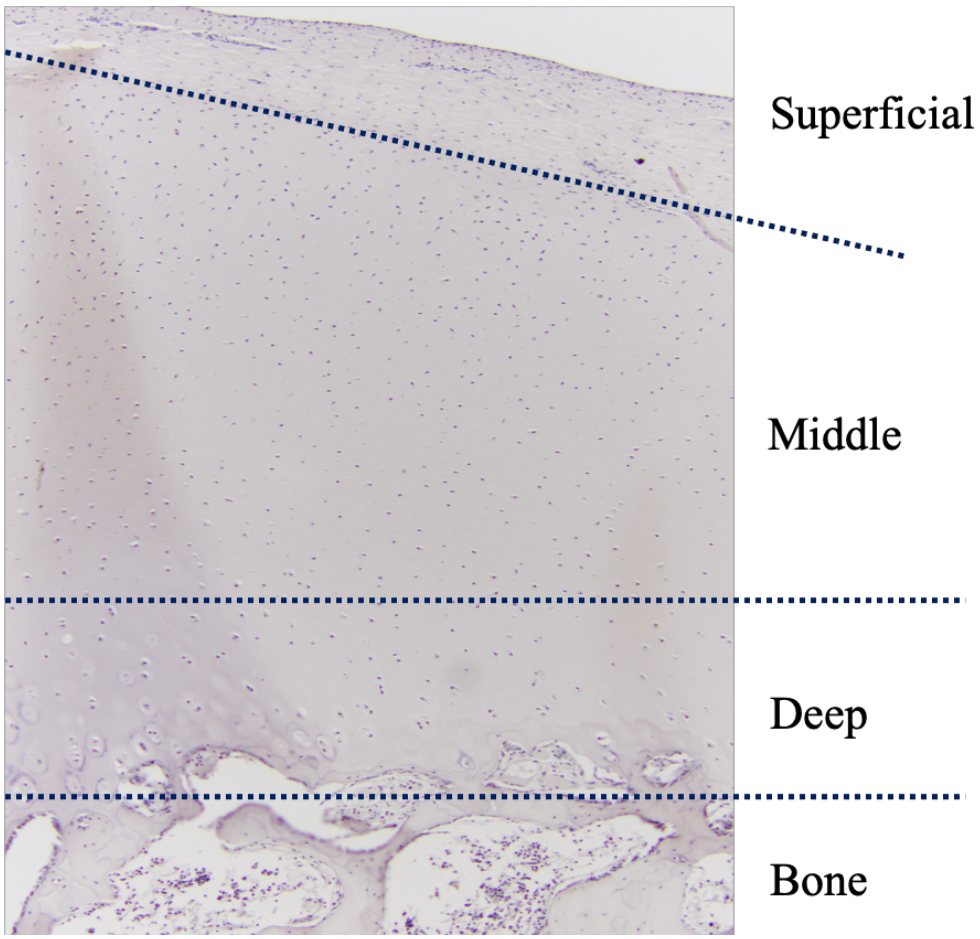

Figure 4. Protein expression for osteochondral sections was scored by layer, including superficial, middle and deep cartilage layers, following immunostaining. The scores of two blinded investigators (SAS and SKG) were averaged for data analysis.

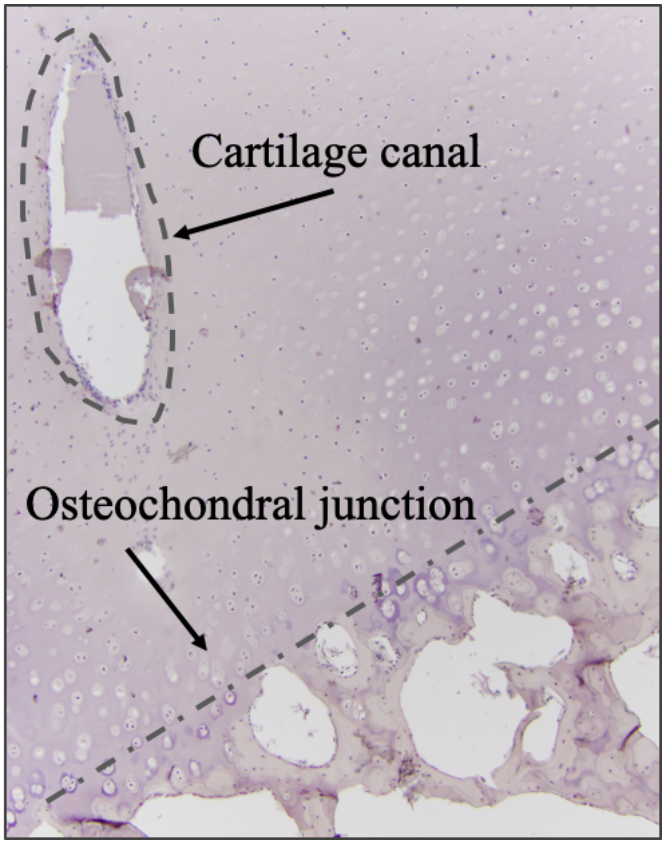

Figure 5. In addition to the cartilage layers, protein expression for osteochondral sections was scored for cells and extracellular matrix surrounding cartilage canals (dashed line) and along the osteochondral junction (dashed and dotted line), following immunostaining. The scores of two blinded investigators (SAS and SKG) were averaged for data analysis.

Chapter 4: Results

Real-time quantitative PCR

Results prior to multiple testing adjustments showed significantly decreased gene expression of Collagen type IIB (p=0.0159**)**, Aggrecan (p=0.0345), and SOX-9 (p= 0.0433) in chondrocytes surrounding the cartilage canals of early osteochondrosis-affected samples (Figure 5, Table 3). In chondrocytes along the osteochondral junction, there was downregulation of expression for Lubricin (p=0.0175) and Collagen type IIB (p=0.0433**)** in osteochondrosis samples (Figure 6, Table 4). However, after performing a false discovery rate test these values were no longer considered as significant findings. There was no difference in gene expression for Collagen type I, Collagen type X, Osteocalcin, SOX-5, TGF-β3, MMP-1, MMP-3, bFGF, COMP, Jagged2, Ephrin-β2, Gremlin1, PRG4, Runx2, or Aggrecanase between OC affected and normal samples using a statistically significant p-value of 0.05 with a corrected significance level of *P*-values ≤ q* =0.0055.

Immunohistochemistry

Protein expression for osteocalcin was observed throughout the extracellular matrix and cells of each cartilage layer (superficial, middle, and deep), surrounding cartilage canals, along the osteochondral junction, and bone (Table 5,6,7,8).

Sporadic osteocalcin expression was observed as mild to moderate staining within chondrocytes throughout all layers of articular cartilage. Protein expression of osteocalcin was confined mainly to osteoblasts and chondroclasts in newly formed bone, as well as in chondrocytes of the deep cartilage layer and along the osteochondral junction. No notable extracellular matrix staining was noted. No significant difference in osteocalcin protein expression was apparent between OC and normal cartilage samples. No statistically

significant differences were seen in HSCORE values for osteocalcin in OC affected cartilage samples versus normal controls for either extracellular matrix or cells within the cartilage layers, surrounding the cartilage canals, along the osteochondral junction, and within bone (Figure 7).

Protein expression for SOX-9 was observed throughout the extracellular matrix and cells of each cartilage layer (superficial, middle, and deep), surrounding cartilage canals, along the osteochondral junction, and bone. Immunostaining for SOX-9 was mild within chondrocytes of each of the cartilage layers. Mild staining was noted sporadically in chondrocytes along the osteochondral junction. Protein expression for SOX-9 was found within osteoblasts and chondroclasts of newly formed bone. Mild immunostaining was observed in chondrocytes surrounding the cartilage canals. No subjective difference was noted in protein expression between OC and normal osteochondral samples. No statistically significant differences were seen in HSCORE values for SOX-9 in OC affected cartilage samples versus normal controls for either extracellular matrix or cells within the cartilage layers, surrounding the cartilage canals, along the osteochondral junction, and within bone (Figure 8).

Protein expression for PRG4 (Lubricin) was observed throughout the extracellular matrix and cells of each cartilage layers (superficial, middle, and deep), surrounding cartilage canals, along the osteochondral junction, and bone. Immunostaining within the extracellular matrix was observed for Lubricin sporadically within the superficial and middle cartilage layers. Lubricin protein expression was found mainly within the deep cartilage layer, along the matrix interface of cartilage and bone in lacunae formed by chondroclasts. Mild to moderate expression was observed within the extracellular matrix surrounding cartilage canals. No extracellular matrix expression was observed within

bone. Within cells, protein expression was observed throughout the cartilage layers, mainly expressed within cells of the deeper cartilage layer. Subjectively, OC affected cartilage had greater immunostaining of chondrocytes along the cartilage canals than that of normal osteochondral samples. Expression was also observed along the osteochondral junction, with no expression observed within bone cells (Figure 10). No subjective difference was noted in protein expression between OC and normal osteochondral samples. No statistically significant differences were seen in HSCORE values for lubricin in OC affected cartilage samples versus normal controls for either extracellular matrix or cells within the cartilage layers, surrounding the cartilage canals, along the osteochondral junction, and within bone (Figure 9).

Protein expression for Collagen type IIB was observed throughout the extracellular matrix and cells of each cartilage layers (superficial, middle, and deep), surrounding cartilage canals, along the osteochondral junction, and bone. Collagen type IIb immunostaining did not follow the expected pattern and degree of staining. This protein is the primary collagen type found within the extracellular matrix and strong expression was expected throughout the cartilage matrix. Collagen type IIB protein expression was not as vivid as expected throughout the cartilage matrix. No statistics were performed for Collagen type IIB due to poor immunostaining which affected observer's ability to perform HSCOREs.

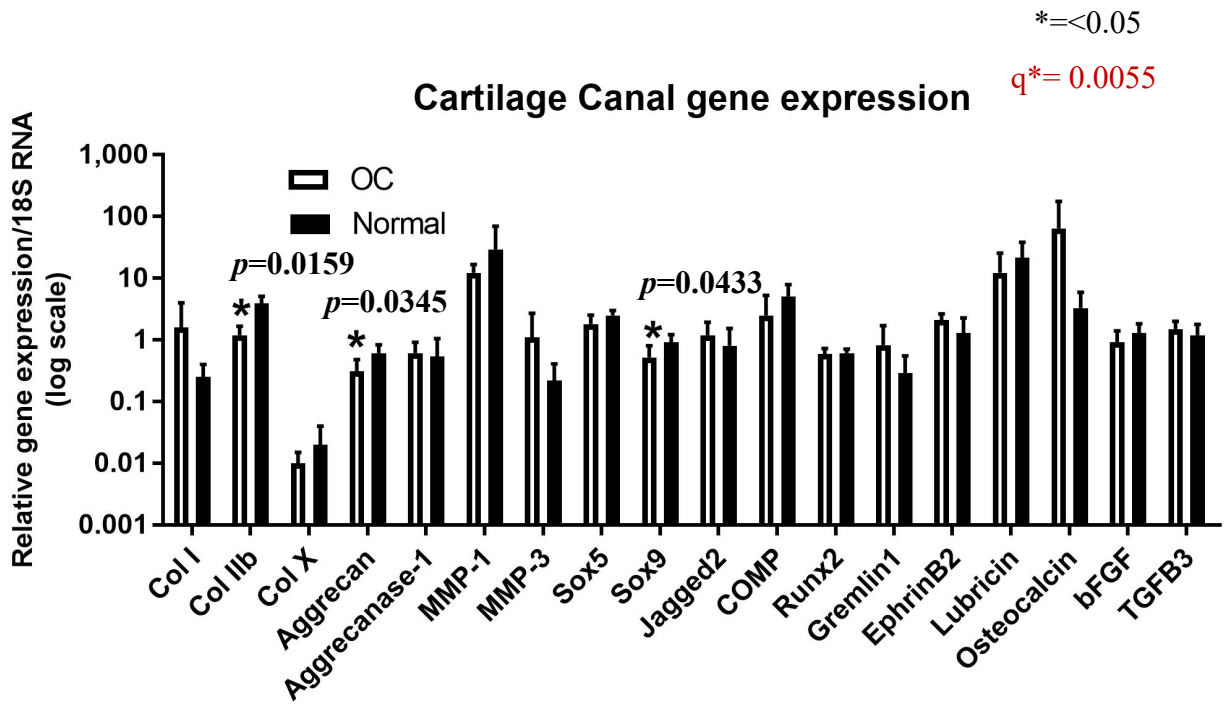

Figure 6. Gene expression of laser-captured cartilage canal chondrocytes using real-time PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18S RNA. There was a trend for downregulation of Collagen type IIB, Aggrecan, and SOX-9 in early OC samples compared to normal controls.

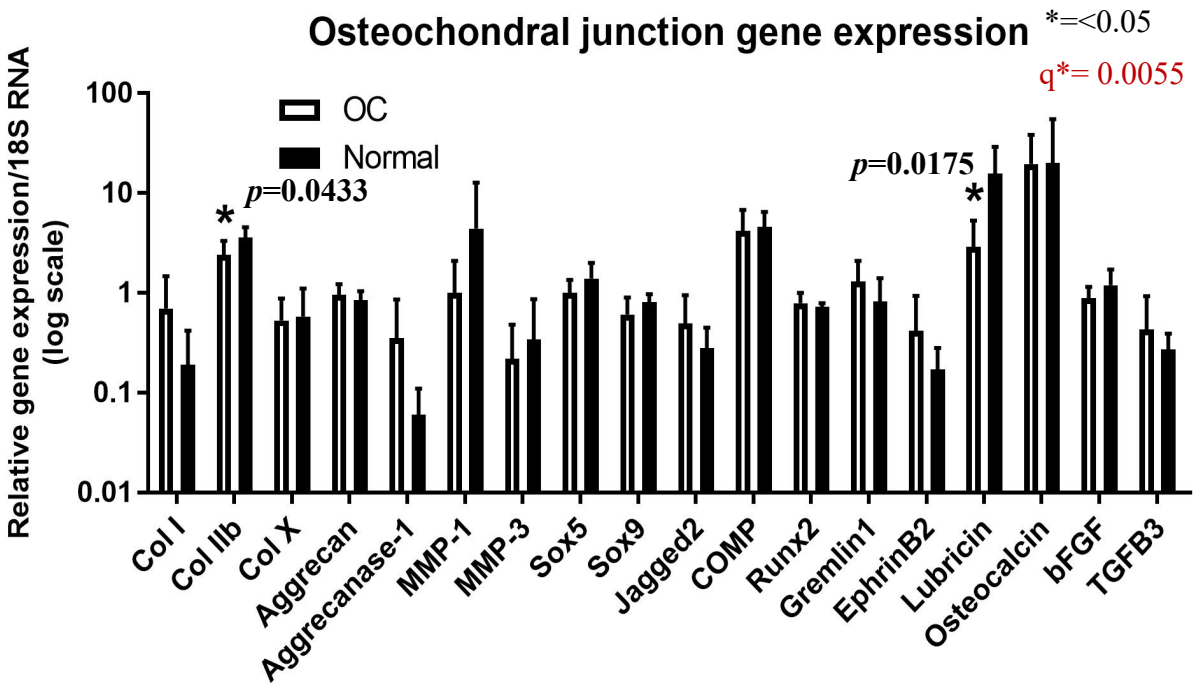

Figure 7. Gene expression of laser-captured osteochondral junction chondrocytes using real-time PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18S RNA. There was a trend for downregulation of collagen type IIB and lubricin in early OC compared to normal controls.

Table 3. Mean gene expression +/- SEM quantified by real-time PCR in laser captured osteochondral junction chondrocytes from early OC and normal control horses. Significance level for gene expression in chondrocytes along the osteochondral junction was corrected to *q=0.005 using a false discovery rate test.

Gene	Early OC	Normal Control	*P*-value
Collagen Type I	0.70 +/- 0.38	0.19 +/- 0.10	0.2857
Collagen Type IIb	2.37 +/- 0.46	3.64 +/- 0.44	0.0433
Collagen Type X	0.54 +/- 0.18	0.58 +/- 0.24	0.7302
Aggrecan	0.96 +/- 0.14	0.85 +/- 0.08	0.5556
Aggrecanase-1	0.35 +/- 0.25	0.06 +/- 0.02	0.4127
MMP-1	1.02 +/- 0.57	4.43 +/- 3.70	0.9048
MMP-3	0.22 +/- 0.13	0.34 +/- 0.24	0.7937
SOX 5	1.04 +/- 0.18	1.37 +/- 0.27	0.4127
SOX9	0.61 +/- 0.15	0.81 +/- 0.07	0.2857
Jagged 2	0.50 +/- 0.23	0.30 +/- 0.07	0.5556
COMP	4.18 +/- 1.30	4.60 +/- 0.88	0.9048
Runx2	0.79 +/- 0.10	0.74 +/- 0.03	0.9048
Gremlin 1	1.29 +/- 0.39	0.82 +/- 0.26	0.556
Ephrin B2	0.42 +/- 0.26	0.17 +/- 0.05	0.7302
Lubricin/PRG4	2.94 +/- 1.19	15.68 +/- 5.98	0.0175
Osteocalcin	19.49 +/- 9.48	19.97 +/- 15.68	0.4127
bFGF	0.89 +/- 0.13	1.18 +/- 0.23	0.2857
TGF-ß3	0.44 +/- 0.25	0.27 +/- 0.05	0.7778

Table 4. Mean gene expression +/- SEM quantified by real-time RT-PCR in laser captured cartilage canal chondrocytes from early OC and normal control horses. Significance level for gene expression in chondrocytes surrounding cartilage canals was corrected to *q=0.005 using a false discovery rate test.

Gene	Early OC	Normal Control	*P*-value
Collagen Type I	1.58 +/- 1.18	0.25 +/- 0.07	0.7302
Collagen Type IIb	1.18 +/- 0.23	3.92 +/- 0.53	0.0159
Collagen Type X	0.01 +/- 0.002	0.02 +/- 0.01	1.000
Aggrecan	0.31 +/- 0.09	0.61 +/- 0.10	0.0345
Aggrecanase-1	0.61 +/- 0.16	0.54 +/- 0.23	0.7302
MMP-1	12.20 +/- 2.19	29.20 +/- 18.0	0.8333
MMP-3	1.09 +/- 0.80	0.22 +/- 0.08	0.4127
SOX-5	1.81 +/- 0.37	2.46 +/- 0.22	0.4127
SOX-9	0.51 +/- 0.15	0.92 +/- 0.13	0.0433
Jagged 2	1.24 +/- 0.37	0.81 +/- 0.33	0.4127
COMP	3.55 +/- 1.39	4.15 +/- 1.24	0.5556
Runx2	0.59 +/- 0.07	0.61 +/- 0.04	0.9048
Gremlin 1	0.83 +/- 0.44	0.29 +/- 0.12	0.4127
Ephrin B2	2.09 +/- 0.28	1.35 +/- 0.44	0.2857
Lubricin/PRG4	12.25 +/- 6.66	21.41 +/- 7.63	0.413
Osteocalcin	64.40 +/- 56.51	3.32 +/- 1.18	0.5556
bFGF	0.92 +/- 0.25	1.27 +/- 0.24	0.2857
TGF-ß3	1.47 +/- 0.26	1.20 +/- 0.26	0.7302

Table 5. Mean protein expression scores +/- SEM following immunohistochemistry of osteochondral sections from early OC and normal controls. Each site was scored by observing *cells* within this region: 0 (no staining/expression) to 3 (strong staining/expression), and the scores of the two observers (SAS and SKG) were averaged.

Protein	Site	Early OC	Normal Control	*P*-value
SOX9	Cartilage Canal	0.07 +/- 0.04	0.01 +/- 0.01	0.1678
	Osteochondral Junction	0.17 +/- 0.13	0.03 +/- 0.01	0.2611
	Bone	0.40 +/- 0.21	0.03 +/- 0.01	0.0816
Lubricin/PRG4	Cartilage Canal	0.43 +/- 0.19	0.10 +/- 0.08	0.4167
	Osteochondral Junction	0.47 +/- 0.16	0.59 +/- 0.19	0.6370
	Bone	0.04 +/- 0.03	0.26 +/- 0.12	0.2821
Osteocalcin	Cartilage Canal	0.64 +/- 0.23	0.41 +/- 0.19	0.6008
	Osteochondral Junction	0.90 +/- 0.15	0.99 +/- 0.16	0.6813
	Bone	1.63 +/- 0.42	1.47 +/- 0.17	0.7242

Table 6. Mean protein expression scores +/- SEM following immunohistochemistry of osteochondral sections from early OC and normal controls. Each site was scored by observing *extracellular matrix* within this region: (no staining/expression) to 3 (strong staining/expression), and the scores of the two observers (SAS and SKG) were averaged.

Protein	Site	Early OC	Normal Control	*P*-value
SOX9	Cartilage Canal	0.00 +/-0.00	0.00 +/-0.00	1.000
	Osteochondral Junction	0.00 +/- 0.00	0.00 +/- 0.00	1.000
	Bone	0.12 +/- 0.12	0.00 +/- 0.00	0.4286
Lubricin/PRG4	Cartilage Canal	0.24 +/- 0.12	0.32 +/- 0.15	0.5238
	Osteochondral Junction	0.18 +/- 0.11	0.06 +/- 0.06	0.3187
	Bone	0.81 +/- 0.21	0.59 +/- 0.12	0.5135
Osteocalcin	Cartilage Canal	0.00 +/- 0.00	0.00 +/- 0.00	1.0000
	Osteochondral Junction	0.00 +/- 0.00	0.00 +/- 0.00	1.0000
	Bone	0.12 +/- 0.12	0.00 +/- 0.00	0.4286

Table 7. Mean protein expression scores +/- SEM following immunohistochemistry of osteochondral sections from early OC and normal controls. Each cartilage layer was scored by observing *cells* within the layers: 0 (no staining/expression) to 3 (strong staining/expression), and the scores of the two observers (SAS and SKG) were averaged.

Protein	Site	Early OC	Normal Control	*P*-value
SOX9	Superficial	0.20 +/- 0.13	0.21 +/- 0.16	0.8951
	Middle	0.15 +/- 0.13	0.08 +/- 0.05	0.9207
	Deep	0.19 +/- 0.13	0.06 +/- 0.02	0.5451
	Total	0.18 +/- 0.13	0.12 +/- 0.07	0.8498
Lubricin/PRG4	Superficial	0.15 +/- 0.07	0.39 +/- 0.14	0.1462
	Middle	0.10 +/- 0.05	0.23 +/- 0.08	0.3177
	Deep	0.27 +/- 0.12	0.56 +/- 0.19	0.3876
	Total	0.18 +/- 0.07	0.39 +/- 0.11	0.2105
Osteocalcin	Superficial	0.46 +/- 0.12	0.58 +/- 0.25	0.5974
	Middle	0.42 +/- 0.07	0.52 +/- 0.08	0.2158
	Deep	0.92 +/- 0.13	1.03 +/- 0.19	0.8781
	Total	0.60 +/- 0.09	0.71 +/- 0.16	0.8518

Table 8. Mean protein expression scores +/- SEM following immunohistochemistry of osteochondral sections from early OC and normal controls. Each cartilage layer was scored by observing *extracellular matrix* within the layers: 0 (no staining/expression) to 3 (strong staining/expression), and the scores of the two observers (SAS and SKG) were averaged.

Protein	Site	Early OC	Normal Control	*P*-value
SOX9	Superficial	0.71 +/- 0.30	0.28 +/- 0.20	0.1102
	Middle	0.04 +/- 0.04	0.01 +/- 0.01	1.000
	Deep	0.00 +/- 0.00	0.00 +/- 0.00	1.000
	Total	0.25 +/- 0.11	0.09 +/- 0.07	0.1242
Lubricin/PRG4	Superficial	0.44 +/- 0.10	0.79 +/- 0.15	0.1312
	Middle	0.02 +/- 0.02	0.03 +/- 0.03	1.0000
	Deep	0.14 +/- 0.09	0.13 +/- 0.13	0.5385
	Total	0.20 +/- 0.04	0.31 +/- 0.08	0.9414
Osteocalcin	Superficial	1.23 +/- 0.35	0.69 +/ 0.25	0.2634
	Middle	0.17 +/- 0.17	0.00 +/- 0.00	0.4286
	Deep	0.00 +/- 0.00	0.00 +/- 0.00	1.0000
	Total	0.47 +/- 0.13	0.23 +/- 0.08	0.2125

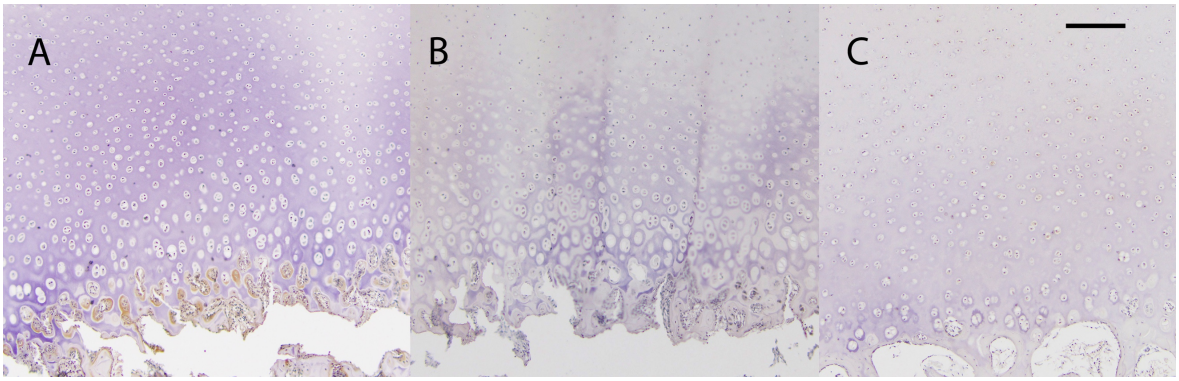

Figure 8: Photomicrographs depicting protein expression for SOX-9 following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate to strong SOX-9 expression in chondrocytes along the osteochondral junction B) Negative control for (A) following substitution of mouse monoclonal SOX-9 antibody with nonimmune serum. C) Osteochondral samples of normal foal. (bar=100µm)

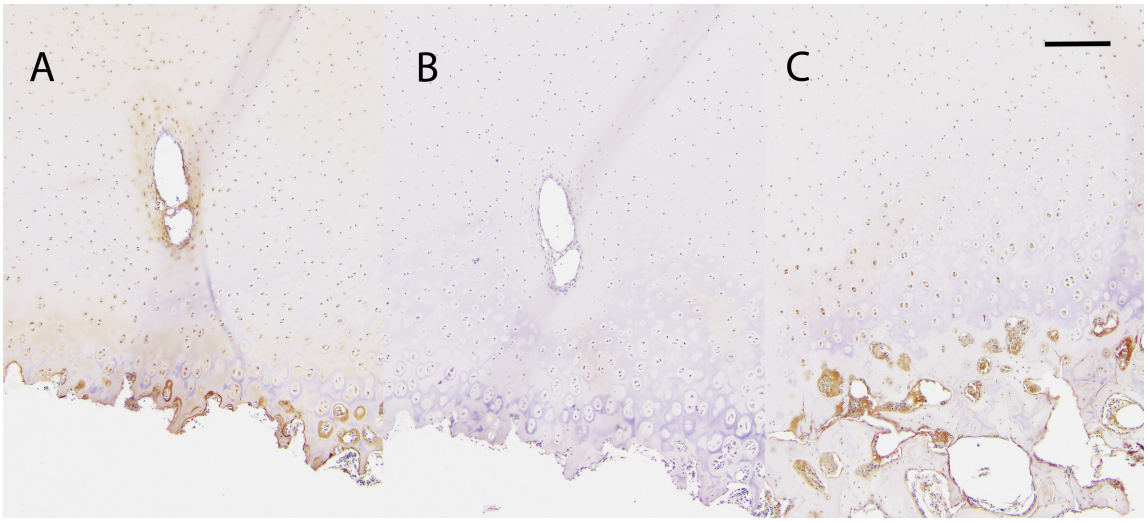

Figure 9: Photomicrographs depicting protein expression for Lubricin following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate to strong lubricin expression in chondrocytes along the osteochondral junction and surrounding cartilage canals with mild to moderate expression throughout the extracellular matrix of the deeper cartilage layers B) Negative control for (A) following substitution of rabbit polyclonal Lubricin antibody with nonimmune serum. C) Osteochondral samples of normal foal. (bar=100µm)

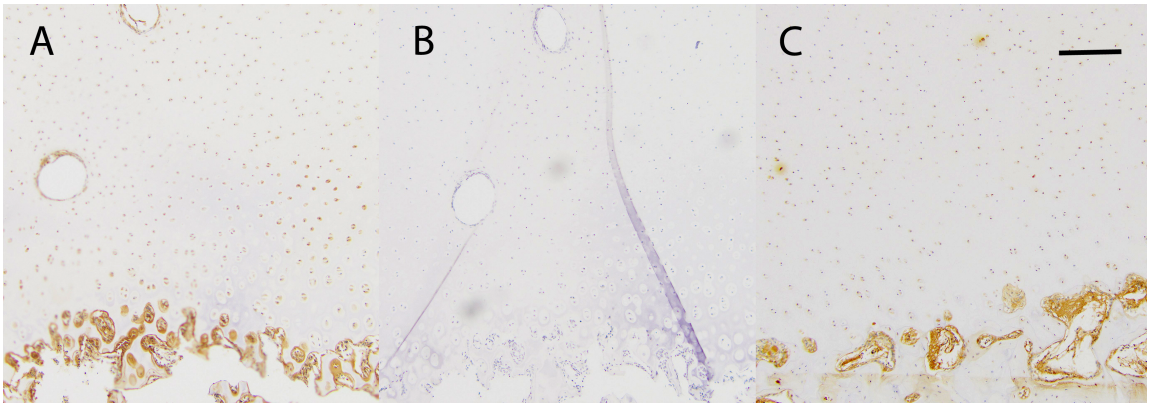

Figure 10: Photomicrographs depicting protein expression for Osteocalcin following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate to strong osteocalcin expression in chondrocytes along the osteochondral junction and in within the deeper cartilage layers B) Negative control for (A) following substitution of mouse monoclonal Osteocalcin antibody with nonimmune serum. C) Osteochondral samples of normal foal showing similar results. (bar=100µm)

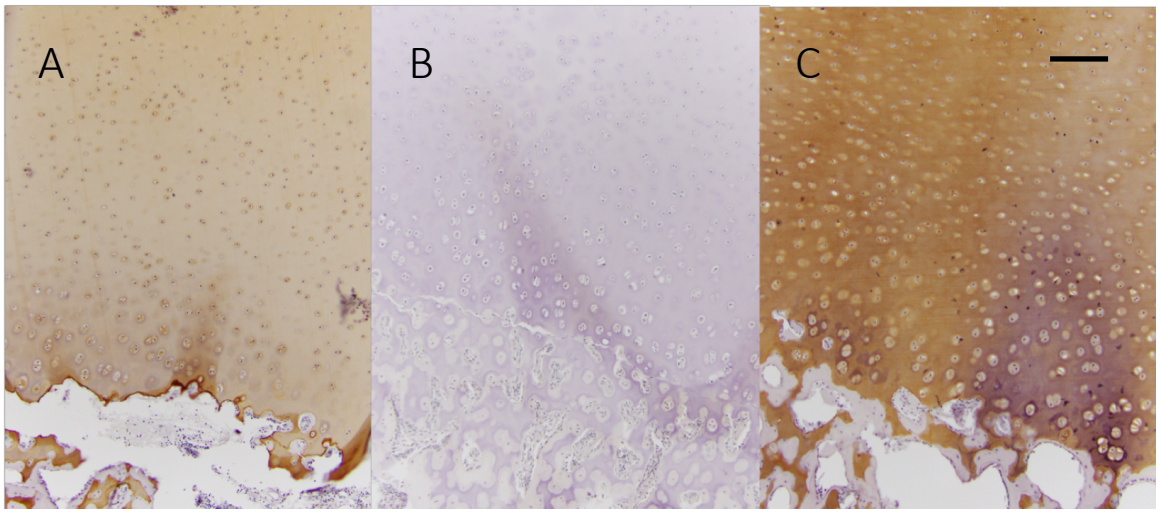

Figure 11: Photomicrographs depicting protein expression for Collagen type IIB following immunohistochemical localization. A) Osteochondral sample of foal with confirmed OC showing moderate to strong Collagen type IIB expression throughout the extracellular matrix of the articular zone with strong expression localized to the hypertrophic zone B) Negative control for (A) following substitution of mouse monoclonal Collagen type IIB antibody with nonimmune serum. C) Osteochondral samples of normal foal showing similar results. (bar=100µm)

Chapter 5: Discussion

The pathogenesis of osteochondrosis remains to be defined in its entirety, although our knowledge of the complex network of interacting molecular pathways continues to grow. Several hypotheses have surfaced with a focus on abnormal extracellular matrix as a potential source of weakness resulting in osteochondrosis dissecans lesions (Laverty and Girard, 2013; Laverty et al., 2000, 2002; Lecocq et al., 2008; van de Lest et al., 2004). We expected to observe differences in gene and protein expression for foals with and without early osteochondrosis. More specifically, we anticipated to find a downregulation of extracellular matrix gene and protein expression and an upregulation of matrix degradation molecules within the osteochondral junction and in cells surrounding the cartilage canals of early osteochondrosis-affected samples. In partial support of our hypothesis, our study revealed a trend for decreased gene expression of collagen type IIB, aggrecan, and SOX-9 in chondrocytes surrounding the cartilage canals of early osteochondrosis-affected samples and of PRG4 (Lubricin) and collagen type IIB in chondrocytes along the osteochondral junction in osteochondrosis samples.

Although not statistically significant, our findings did reveal a trend for downregulation of collagen type IIB gene expression in cells along the osteochondral junction and in those surrounding cartilage canals of osteochondrosis-affected foals. Collagen type IIB has been shown to be altered in the extracellular matrix of early equine osteochondrosis (Laverty et al., 2002; Lecocq et al., 2008) and has a significant effect on tensile strength properties of cartilage. It has been long questioned whether the changes seen in collagen within the cartilage matrix are primary or secondary cellular events. In our study, we discovered a decrease in gene expression of collagen type II in cells along the osteochondral junction and near cartilage canals. This may support the hypothesis that

weakened extracellular matrix in these sites subjected to shearing forces and failure of anastomoses of blood supply may contribute to the high prevalence of osteochondrosis found in these particular sites. These findings are similar to previous studies which have shown a reduction in total type II collagen content and number of collagen crosslinks in osteochondrosis affected foals. However, our findings differ from previous studies in which an increase in collagenase gene expression is accompanied by an increase in collagen degradation (Hurtig et al., 1993; Laverty et al., 2002; Wardale and Duance, 1994). In contrast to previous research, our study evaluating laser capture microdissection samples did not observe the expected upregulation of matrix degradation expression. A previous study evaluating full thickness cartilage samples found an upregulation of MMP-3 and MMP-13 in osteochondrosis-affected samples (Riddick et al., 2012), while we did not find increased MMP-1 or MMP-3 gene expression in surrounding cartilage canals or along the osteochondral junction. This discrepancy is very likely due to differences in sample types (full thickness vs laser captured cells).

Immunohistochemistry scoring was made difficult due to poor staining and difficulties in handling of samples during this procedure. These factors may also to have contributed to the lower intensity staining than what should be expected for collagen type IIB as the primary collagen type for articular cartilage (Mcilwraith, 2016). In addition, immunohistochemistry has been implicated in reduced sensitivity of detecting protein expression (Kim et al., 2016). Immunohistochemistry involves several steps, any number of which may have contributed to the abnormalities found within protein expression of Collagen type IIB. Degeneration of protein and tissue may have occurred during initial sample preparation and fixation, or long-term storage of slides may result in degradation of tissue. For validation of antibodies it is recommended that validation of antibody using

methods other than immunohistochemistry (western blotting, flow cytometry) in order to ensure that optimal detection occurs, which was not performed in our study.

In our study, a trend for reduced aggrecan gene expression was found in chondrocytes surrounding the cartilage canals of OC-affected foals. Aggrecan, a proteoglycan bound to hyaluronic acid, is a critical component for cartilage structure and the function of joints. Due to its expression by chondrocytes, it is often used as an indicator of chondrocyte activity and may play an important role in early mineralization (Hoogen et al., 1999). Early studies have shown that the size and shape of the proteoglycan may become altered in order to allow for new bone formation to occur, but that there is not net loss of aggrecan in normal mineralization (Pool, 1993). Our findings of reduced aggrecan gene expression in chondrocytes surrounding cartilage canals in osteochondrosis affected foals may predicate failure of the cartilage canals and ischemic necrosis (Laverty and Girard, 2013; Olstad et al., 2013).

A trend for SOX-9 gene expression downregulation was found in chondrocytes surrounding the cartilage canals of osteochondrosis-affected foals. SOX-9 plays a critical role in chondrocyte differentiation. It sends an inhibitory signal to chondrocytes entering into hypertrophy, likely to balance the stimulatory effects on maturation of chondrocytes signaled by SOX-5 and SOX-6 (Ikeda et al., 2005). In combination with SOX-5 and SOX-6, it has been shown to directly activate the gene for aggrecan and collagen type IIa, yielding downstream effects on cartilage formation (Lefebvre et al., 2001). The expression of SOX-9 has been noted to decrease in hypertrophic chondrocytes and it has been speculated that the expression of SOX-5 and SOX-6 is reliant upon that of SOX-9. The significance of these findings may be associated with the role of SOX-9 in

contributing to a weakened extracellular matrix along the osteochondral junction, resulting in increased susceptibility to shearing forces in this region.

In osteochondrosis samples, a trend for reduced PRG4 gene expression was found in cells along the osteochondral junction. PRG4 encodes the mucinous glycoprotein lubricin. Lubricin is present in synovial fluid and functions as a boundary lubricant and as a chondroprotective agent and has been shown to delay the progression of OA in rodent models. PRG4 is a highly influenceable gene and its expression is regulated by many growth factors and BMPs. Its downregulation in early osteochondrosis has been speculated to be merely a result of concurrent cell signaling patterns (Yang, 2009).

Although no difference in BGLAP gene expression was apparent in OC samples, immunohistochemistry was performed for osteocalcin. Previous studies have shown that the presence of OC lesions is significantly correlated to elevated serum osteocalcin concentrations in foals (Donabedian et al., 2008). This study indicated that increased bone metabolism due to the presence of osteochondrosis in foals as early as 2 weeks old could be detected before radiographic changes occurred at 5.5 to 11 months of age. By evaluating protein expression of osteocalcin, the aim of the experiment was to gain a better understanding of the molecular changes occurring within the early growth period as we see regression of cartilage canals (Olstad et al., 2008; Ytrehus et al., 2004). In our study, protein expression of osteocalcin was confined mainly to osteoblasts and chondroclasts in newly formed bone, as well as in chondrocytes of the deep cartilage layer and along the osteochondral junction. No observable or statistically significant differences were noted between OC affected and normal control osteochondral samples. This may be contributed to the reduced sensitivity of immunostaining in the detection of protein expression (Riddick et al., 2012).

Although trends for differences were observed in gene expression when osteochondrosis and normal cartilage samples were compared, these suggestive trends observed in gene expression were not mirrored in our protein expression results. Once again we observed that variations can be seen between gene and protein expression as described in other studies (Riddick et al., 2012). Our study was conducted using samples collected from foals ranging in age from 0-6 months. This critical time period for growth has been shown to be significant in the development of histological lesions compatible with osteochondrosis in a multitude of studies (Carlson et al., 1995; Olstad et al., 2008). However, taking into consideration the highly dynamic nature of these molecular processes it is impossible to eliminate the temporal variances at the time samples were collected. By evaluating specific sites within the trochlear ridges of the femoral condyle, and with the aid of laser capture microdissection, it was made possible to evaluate very specific areas within the cartilage including cells surrounding the cartilage canals and along the osteochondral junction in order to eliminate spatial discrepancies.

Limitations of the study included a targeted rather than comprehensive evaluation of genes expressed in the extracellular matrix, mainly due to limited amount of cDNA from laser-captured samples. In addition, only two cell populations were studied due to having a limited time to complete the laser capture procedure prior to sample degradation. It is also important to note that gene and protein expression studies do not address longitudinal changes or activity of molecules within the region. The small number of samples likely contributed to type I error seen in our initial statistical results, which after using a false discovery rate and Bonferroni correction indicated that the statistical significance found within these values were likely false. We did not evaluate protein expression for each gene due to financial constraints, limiting our ability to catch

any discrepancies. As with many studies, our results do not show a clear connection between gene and protein expression and presence of disease.

Chapter 6: Conclusion

In summary, our specific results may contribute to the findings that a weakened extracellular matrix, particularly in areas susceptible to shearing forces along the osteochondral junction and in areas prone to ischemic necrosis near the cartilage canals, contributes to the pathophysiology of OC. Future studies may be directed at incorporating tensile strength tests in order to evaluate the biomechanical significance of these findings. The process of endochondral ossification requires intricate molecular signaling pathways in order for cartilage templates to properly form into long bones or the main structural components of joints. The impact of improper formation of bone from cartilage may result in a clinical disease in horses, known as osteochondritis dissecans, in which bone or cartilage flaps create an inflammatory state within the joint. The disease has significant economic impact, limitations of the animal as an athlete, and results in a reduction of the available gene pool. By gaining an understanding of its pathogenesis, early diagnosis may provide management options to prevent formation of clinical lesions.

Although not all encompassing, this study is a comprehensive evaluation of gene and protein expression comparisons between normal and early osteochondrosis-affected osteochondral samples. The information gained from this study thus fills a gap in our knowledge base by providing a description of molecular activity during this critical growth period. Samples used in this study contributed to several previous studies evaluating gene and protein expression in foals with and without osteochondrosis, which when evaluated together paint a

much more comprehensive picture of the complex molecular events occurring during this process (Kinsley and Kinsley, 201510; Riddick et al., 2012; Semevolos et al., 2018) (Table 9, 10). To date, there is no current model for studies in endochondral ossification and osteochondrosis in children. The information gathered in this study, in addition to facilitating a better understanding of OC in horses, could pave the way for a model for this condition in humans.

Table 9. Osteochondral samples were used for several studies evaluating gene and protein expression in foals with and without osteochondrosis, ranging in age from 1-6 months of age. This table is a comprehensive overview of significant gene expression findings following real time q-PCR within each study.

Study	Author	Target genes	Cartilage canals	Osteochondral Junction	Whole Cartilage
"Gene and protein expression of cartilage canal and osteochondral junction chondrocytes and full-thickness cartilage in early equine osteochondrosis"	Riddick, *et al*. 2012	Ihh, PTH-rP, VEGF, PDGF-A, MMP-3, MMP-13	↑ MMP-13 ↑ PDGF-A	↑ PDGF-A	↑ MMP-3, MMP-13 ↑ Ihh
"Wnt/β-catenin signaling of cartilage canal and osteochondral junction chondrocytes and full thickness cartilage in early equine osteochondrosis"	Kinsley *et al*., 2015	β-catenin, Wnt-11, Wnt 4, Wnt-5b, Dkk-1, Lrp6, Wif-1, Axin1, SC-PEP, RARγ	↓ Wnt-11 ↑ β-catenin, Wnt-5b, Dkk-1, Lrp6, Wif-1, Axin1, and SC-PEP	↑ β-catenin ↑ SC-PEP	No significant findings
"Expression of pro-apoptotic markers is increased along the osteochondral junction in naturally occurring osteochondrosis"	Semevolos *et al*., 2018	Caspase-3, Caspase-8, Caspase-10, Fas, Bcl-2, BAG-1, TNFα, Cytochrome *C*, Thymosin-β10	↑ Fas	↑ Caspase-10, Fas, Cytochrome *C*, and Thymosin-β10	Not evaluated
"Role of cartilage and bone matrix regulation in early equine osteochondrosis"	Grissom, 2018	Collagen types I, IIB, and X, Aggrecan, Aggrecanase-1, MMP-1, MMP-3, SOX 5, SOX 9, Jagged 2, COMP, Runx2, Gremlin 1, Ephrin B2, Lubricin (PRG4), Osteocalcin, bFGF, and TGF-ß3	Downward trend for Collagen type IIB, Aggrecan, SOX-9	Downward trend for Collagen type IIB, Lubricin	Not evaluated

Table 10. Osteochondral samples were used for several studies evaluating gene and protein expression in foals with and without osteochondrosis, ranging in age from 1-6 months of age. This table is a comprehensive overview of significant protein expression findings following immunohistochemistry within each study.

Study	Author	Target genes	Cartilage canals	Osteochondral Junction	Whole Cartilage
"Gene and protein expression of cartilage canal and osteochondral junction chondrocytes and full-thickness cartilage in early equine osteochondrosis"	Riddick, *et al*. 2012	Ihh, PTH-rP, VEGF, PDGF-A, MMP-3, MMP-13	↑ MMP-13 ↑ PDGF-A	↑ PDGF-A	↑ MMP-3, MMP-13 ↑ Ihh
"Wnt/β-catenin signaling of cartilage canal and osteochondral junction chondrocytes and full thickness cartilage in early equine osteochondrosis"	Kinsley *et al*., 2015	β-catenin, Wnt-11, Wnt 4, Wnt-5b, Dkk-1, Lrp6, Wif-1, Axin1, SC-PEP, RARγ	↓ Wnt-11 ↑ β-catenin, Wnt-5b, Dkk-1, Lrp6, Wif-1, Axin1, and SC-PEP	↑ β-catenin ↑ SC-PEP	No significant findings
"Expression of pro-apoptotic markers is increased along the osteochondral junction in naturally occurring osteochondrosis"	Semevolos *et al*., 2018	Caspase-3, Caspase-8, Caspase-10, Fas, Bcl-2, BAG-1, TNFα, Cytochrome *C*, Thymosin-β10	↑ Fas	↑ Caspase-10, Fas, Cytochrome *C*, and Thymosin-β10	Not evaluated
"Role of cartilage and bone matrix regulation in early equine osteochondrosis"	Grissom, 2018	Collagen types I, IIB, and X, Aggrecan, Aggrecanase-1, MMP-1, MMP-3, SOX 5, SOX 9, Jagged 2, COMP, Runx2, Gremlin 1, Ephrin B2, Lubricin (PRG4), Osteocalcin, bFGF, and TGF-ß3	Downward trend for Collagen type IIB, Aggrecan, SOX-9	Downward trend for Collagen type IIB, Lubricin	Not evaluated

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