AN ABSTRACT OF THE THESIS OF

Marc A. Kinsley for the degree of Master of Science in Veterinary Science presented on June 4, 2013

Title: Molecular Characterization of Wnt/β-catenin Signaling in Early Equine Osteochondrosis

Abstract approved:

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Stacy A. Semevolos

Abstract: The objective of this study was to elucidate the expression of signaling molecules associated with the Wnt signaling pathway in cartilage of prepubescent foals and determine their association with chondrocytes located at the osteochondral junction and cartilage canals. Our hypothesis was that increased expression of the components of the Wnt signaling pathway in chondrocytes of the osteochondral junction and cartilage canals would be found in early osteochondrosis (OC) lesions when compared to normal controls. Chondrocytes surrounding cartilage canals and the osteochondral junction were captured using laser capture microdissection from cartilage samples collected from 6 foals with early OC and 8 normal controls. Equine-specific β-catenin, Wnt-4, Wnt-5b, Wnt-11, Dkk-1, Lrp4, Lrp6, Axin1, WIF-1, Sfrp1, Sfrp3, Sfrp5, RARgamma, SC-PEP and 18S mRNA expression levels were evaluated by two-step real-time quantitative PCR. Spatial tissue protein expression of β-catenin, Wnt-11, Wnt 4, and Dkk1 was determined by immunohistochemistry. There was significantly decreased Wnt-11 and increased β-catenin, Wnt-5b, Dkk1, Lrp6, WIF-1, Axin1, and SC-PEP gene expression in early OC

chondrocytes along the cartilage canals compared to controls. There was also significantly increased β-catenin and SC-PEP gene expression in early OC chondrocytes along the osteochondral junction compared to normal controls. Wnt 11 showed significantly decreased protein expression in the superficial cartilage layer and Dkk1 showed significantly decreased protein expression in the deep cartilage. Increased gene expression of β-catenin, Wnt 5b, WIF-1, SC-PEP, Axin1, Dkk1, and Lrp6 provides evidence that canonical Wnt signaling is altered in early osteochondrosis of horses and may be associated with disease pathogenesis.

Molecular Characterization of Wnt/β-catenin Signaling in Early Equine Osteochondrosis

By

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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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Marc A. Kinsley, Author

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Chapter 1: Introduction

Multicellular organisms arise by a relatively slow process of progressive change. In nearly all cases, the development of a multicellular organism begins with a single cell which divides to produce all cells of the body. Development is coordinated and controlled through secreted signaling molecules and continues after birth. These signaling molecules bind to specific receptors that activate cascades of events to increase or decrease transcription controlled by specific transcription factors. During development, a limited number of pathways provide critical cell to cell communication required to regulate processes such as cell proliferation, survival and cell differentiation activities (Gordon and Nusse, 2006). Regulation of signaling is crucial and any changes to any given signal transduction pathway can lead to devastating results such as developmental defects or disease later in life. The Wingless-related integration site (Wnt) signaling pathway is one such critical pathway involved in many developmental and homeostatic processes and has been demonstrated in the pathogenesis of a variety of diseases (Prunier et al., 2004)

Wnt Signaling

The vertebrate Wnt family is a group of highly conserved growth factors responsible for important cell development and homeostasis (Miller et al., 2001). Wnts are secreted glycoproteins that act by binding Frizzled (Fz), a family of seven-pass transmembrane receptors, and Low density lipoprotein receptor-related protein cell

surface receptors (Lrp). There are a total of 19 Wnt signaling molecules and 11 Fz receptors currently known. Upon a specific Wnt binding to a specific frizzled receptor, a signal is transduced via either the canonical β-catenin-lymphoid enhancer binding factor 1/ T cell-specific transcription factor (LEF/TCF) pathway, non-canonical Ca^{2+}-calmodulin-PKC pathway, or JNK-dependent pathway (Figure 1) (Miller, 2001; Pandur et al., 2002; Gordon and Nusse, 2006; Milat and Ng 2009).

Of the three Wnt pathways, the canonical pathway has been best characterized. In the absence of Wnt binding, β-catenin forms complexes with glycogen synthase kinase 3β, axis inhibitory protein (Axin), and adenomatous polypolis coli (APC). When associated with this complex of proteins, also known as the "destruction complex", glycogen synthase 3β phosphorylates β-catenin in multiple sites and allows β-catenin to dissociate from the destruction complex. Phosphorylated β-catenin is recognized, ubiquitylated, and is directed for proteasome assisted degradation (Gordon and Nusse, 2006). This keeps cytoplasmic levels of β-catenin low and prevents β-catenin induced transcription. When a Wnt signal is present, it binds to a Fz receptor, a 7 transmembrane serpentine receptor, complexed to a low density lipoprotein receptor related protein (LRP), a single pass transmembrane protein required for signal transduction. Upon binding the Wnt signal, Fz interacts with Disheveled, a scaffolding protein, and induces Disheveled dependent phophorylation of the cytoplasmic tail of LRP (Krestler and Kuhl, 2008). Axin, with the other components of the destruction complex, is recruited to the cell membrane and binds to phosphorylated LRP. The cytoplasmic tail of LRP facilitates the dissociation of β-catenin from the destruction complex. Glycogen synthase 3β can no longer phosphorylate β-catenin and β-catenin accumulates in the cytoplasm. β-catenin

can then translocate into the cell's nucleus and co-activate LEF/TCF transcription factors, resulting in the expression of target genes(Figure 1; Gordon and Nusse 2006; Milat and Ng 2009).

The non-canonical Ca^{2+}-calmodulin-PKC pathway is less well characterized, but it is known that binding of different Wnts to different Frz receptors than those in the canonical pathway leads to activation of a G-protein. Activation leads to downstream activation of phospholipase C. Phospholipase C causes the formation of diacylglycerol and inositol-3-phosphate which in turn activate protein kinase C. Activated protein kinase C is able to phosphorylate and inhibit TCF transcription factors thereby decreasing canonical signaling and also activates the NF-AT transcription factor (Miller, 2001; Krestler and Kuhl, 2008).

The JNK-dependent pathway is the least characterized of the currently known Wnt signaling pathways. In this pathway, Wnt binds to a Fzd receptor and activates the Rho family of GTPases downstream of Disheveled. Rho GTPases act as molecular switches similar to other GTP-binding proteins and are active when bound to GTP. They affect many aspects of cell behavior including actin cytoskeleton dynamics, transcriptional regulation, cell cycle progression, and membrane trafficking (Kjoller and Hall 1999). In Wnt signaling, most of the affect seen through activation of Rho GTPases is through their ability to activate JNK transcription factors (Krestler and Kuhl, 2008).

Although most of the research into Wnt signaling describes these three separate pathways, there is a push for it to be considered more of a network with all the pathways interacting to control signal transduction (Kestler and Kuhl 2008). In support of this network, the same Wnt signaling molecule can activate different signaling pathways. For

example, Wnt 5a can activate both the non-canonical and canonical signaling pathways (He et al., 1997; Weeraratna et al., 2002). In addition, the same Fz receptors have been shown to activate different pathways and Disheveled is involved in both canonical signaling and the JNK-dependent pathways (Boutros et al., 1998). JNK- dependent activation has been shown to be dependent on the PKC activation that occurs in the Ca^{2+}-calmodulin-PKC pathway and PKC is able to inhibit β-catenin signaling through phosphorylation of TCF transcription factors (Milat and Ng 2008). So, although it is easier to understand, describe, and visualize Wnt signaling as 3 separate pathways, it is important to realize that Wnt signaling is more complex and work still remains to elucidate all the interactions that occur in Wnt signaling.

Due to its importance in vertebrate development and homeostasis, Wnt signaling is under strict control via a multitude of negative inhibitors including Wnt inhibitory factor-1 (Wif1), Dickkopf-1 (Dkk1), Axis inhibition protein 1 (Axin 1), glycogen synthase 3β, adenomatous polypolis coli (APC), and secreted frizzled related proteins 1, 3 and 5 (Sfrp 1,3,5) (Hsieh et al., 1999; Kawano and Kypta, 2003; Milat and Ng 2009). Wif 1 and the Sfrps are secreted similar to Wnt proteins and are able to competitively bind to Wnts and prevent them from binding to their appropriate Fz receptor. In comparison to other secreted antagonists, Dkk1 functions to inhibit Wnt signaling by binding to LRPs. Dkk1 bound to LRP leads to internalization of LRPs and transport to the lysosome for degradation. By decreasing its availability as a co-receptor to Fzd, Dkk1 decreases canonical signaling. Axin1, glycogen synthase 3β, and APC work in concert to inhibit β-catenin intracellularly by phosphoylating β-catenin marking it for proteosomal degradation.

Although Wnt signaling has been shown to be important in many developmental processes, skeletogenesis studies have provided the best understanding of this pathway. Wnt signaling has been shown to be a key regulator in chondrogenic vs. osteogenic mesenchymal stem cell fate and is an important pathway in endochondral ossification.

Endochondral Ossification

In all species, bone is formed through one of two processes. Bones of the skull or flat bones form through the process of intramembranous ossification. Long bones form through a process known as endochondral ossification. Endochondral ossification is defined as the process by which growing cartilage is systematically replaced by bone to form the growing skeleton. Embryonically, cartilage models are formed through condensation of mesenchymal cells, followed by their differentiation into chondrocytes. This differentiation is under the control of many paracrine and autocrine signaling molecules. Depending on the amount, type and timing of these signals, mesenchymal cells can either differentiate into chondrocytes or osteoblasts within the growing bone. In order for appropriate endochondral ossification to occur, these signals must be appropriately coordinated.

Growing cartilage is found in two locations at each end of developing bone: the growth plate and the articular-epiphyseal growth cartilage, which drive expansion of the primary and secondary centers of ossification, respectively (Mackie et al., 2008). Chondrocytes in growth cartilage are arranged in morphologically distinct zones that

reflect changes in the functional state of the cells (Byers and Brown, 2006). The zone furthest from the ossification front is the zone of resting chondrocytes, which are surrounded by extracellular matrix and serve as precursors to the adjacent zone of proliferation. In the zone of proliferation at the articular-epiphyseal complex, round proliferating chondrocytes impinge upon one another leading to chondrocyte flattening and the appearance of multicellular clusters. Following proliferation, chondrocytes pass through a transition stage and are called prehypertrophic chondrocytes. These chondrocytes go through a sequential process of proliferation and production of extracellular matrix (Semevolos and Nixon 2007; Ytrehus et al., 2007; Mackie et al., 2008; Day and Yang 2008; Milat and Ng 2009). These chondrocytes further differentiate and hypertrophy, forming the hypertrophic zone of cartilage. Hypertrophic chondrocytes contain numerous membrane bound matrix vesicles containing annexins, phosphate transporters, and alkaline phophatase that are released. The matrix becomes mineralized through the deposition of hydroxyapatite. Alkaline phosphatase, a marker or hypertrophic chondrocytes, is thought to be critical in matrix mineralization. Hypertrophic chondrocytes get large and outgrow their space within the mineralized matrix and begin expressing matrix metalloproteinase 13 (MMP13). MMP13 degrades the extracellular matrix immediately surrounding the hypertrophic cells, providing hypertrophic chondrocytes more space to further hypertrophy. In the final stage of endochondral ossification, hypertrophic chondrocytes die and empty lacuna are left behind. Septae between lacunae are broken down by chondroclasts and the mineralized matrix is broken down by MMP13 allowing for invasion by vessels from the perichondrium and bone is formed in the lacuna by osteoblasts transported via vascular

invasion (Figure 2) (Semevolos and Nixon 2007; Ytrehus et al., 2007; Mackie et al., 2008; Day and Yang 2008; Milat and Ng 2009). The process through which hypertrophic chondrocytes die has been the subject of debate. The two main hypotheses include hypertrophic chondrocyte apoptosis or autophagic death. Support for apoptosis is provided the detection of DNA strand breaks and other factors known to be associated with apoptosis. The process however, does not truly mimic the way other cells undergo apoptosis and may autophagocytize themselves (Mackie et al., 2008; Milat and Ng 2009).

The final product of endochondral ossification at the epiphyseal-articular complex is mature hyaline articular cartilage attached to bone. Similar to growth cartilage, articular cartilage maintains the same arrangement of zones as existed prior to its conversion to bone (Byers and Brown, 2006). The most superficial zone is composed of elongated chondrocytes oriented with collagen fibrils parallel to the direction of the articular surface (Flirk et al., 2007). Deep to the superficial zone is the middle or transitional zone. This zone is composed of haphazardly spaced and rounded chondrocytes with obliquely oriented collagen fibrils (Flirk et al., 2007). The deep zone is the next distinguishable region. In this zone, spherically shaped chondrocytes are arranged in columns and larger collagen fibrils are arranged perpendicular to the articular surface (Flirk et al., 2007). Finally, the deepest zone, the calcified zone, is characterized by calcified cartilage (Byers and Brown, 2006; Flirk et al., 2007). The junction between the deep zone and calcified zone is known as the tidemark and can be seen histologically in adult cartilage.

Cell Signaling in Endochondral Ossification

The sequential process of endochondral ossification is controlled by numerous autocrine and paracrine signals including growth hormone (GH), insulin like growth factor-1 (IGF-1), thyroid hormone (TH), parathryroid hormone (PTH), platelet derived growth factor (PDGF), Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTHrP), bone morphogenic protein (BMP), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), the Wnt family of proteins, and retinoic acid pathway (Kieswetter et al., 1997; Tamamura et al., 2005; Semevolos et al., 2006; Mackie et al., 2008; Milat and Ng 2009; Yasuhara et al., 2010). These signaling molecules must be coordinated and controlled for chondrocyte differentiation to proceed normally and it appears that the Wnt signaling pathway may be the final common pathway leading to bone formation (Milat and Ng, 2009).

Growth hormone, a systemic regulator of longitudinal bone growth, is secreted by the anterior portion of the pituitary gland. Growth hormone stimulates increased deposition of protein by the chondrocytic and osteogenic cells, increases the rate of reproduction of these cells, and has a specific effect of converting chondrocytes into osteogenic cells, causing the deposition of new bone. Most of the effects of growth hormone are due to increased local secretion of insulin-like growth factor -1 (IGF-1) by growth plate chondrocytes induced by growth hormone. Growth hormone receptors and IGF-1 receptors are present at all stages of chondrocyte differentiation (Hunziker et al., 1993). Mice with reduced circulating IGF-1 have decreased longitudinal growth (Yakar et al., 2002) and in mice with inactivated growth hormone receptors, systemic administration of IGF-1 can prevent abnormalities in growth (Guevara-Aguirre et al.,

1997, Sims et al., 2000). A single IGF-1 allele is a major determinant of small size in dogs (Sutter el al., 2007). Thus, growth hormone and IGF-1 are important factors influencing endochondral ossification.

Thyroid hormone, another systemic regulator of skeletal growth, has been shown to be involved in growth plate formation and chondrocyte hypertrophy. Thyroid hormone and triiodothyronine (T3) induce morphological changes in chondrocytes consistent with hypertrophy, increasing expression of collagen type X and alkaline phosphatase activity (Bohme et al., 1992; Bullock and Reddi, 1994). These molecular changes are most likely due to thyroid hormone's ability to increase Wnt/ β-catenin signaling (Wang et al., 2009). Thyroid hormone is also required for normal levels of growth hormone receptor, IGF-1, and IGF-1 receptor expression in growth plate chondrocytes (O'Shea et al., 2005) and in-vitro, T3 induces death of hypertrophic chondrocytes via non-apoptotic modes identical to those observed in vivo (Ahmed et al., 2007) displaying their importance in endochondral ossification.

Parathyroid hormone (PTH) is the principle regulator of calcium homeostasis in vertebrates and intermittent administration of PTH can induce new bone formation (Jiang et al., 2003). PTH null mice have enlarged hypertrophic zone at their growth plates, reduction cartilage matrix mineralization, reduced vascularization and bone lengths were reduced (Miao et al., 2002). PTH overlaps with many pathways involved in endochondral ossification and has been shown in bone to increase canonical Wnt signaling in osteoblasts (Tobimatsu et al., 2006; Wan et al., 2008). Dkk1 inhibits PTH's effects on bone formation in a concentration dependent manner suggesting that intact Wnt signaling is required for PTH's downstream effects on endochondral ossification (Guo et al., 2010).

Indian hedgehog (Ihh) signaling pathways regulate chondrocyte hypertrophy and maturation. In developing cartilage, Ihh is expressed in prehypertrophic chondrocytes and is a major driving force for chondrocyte proliferation. Ihh controls the pace of chondrocyte hypertrophy by activating the expression of parathyroid hormone related peptide (PTHrP), which acts as an inhibitor of chondrocyte hypertrophy forming a negative feedback loop (Vortkamp el al., 1996; Day et al., 2005). PTHrP is expressed by perichondrial cells and early proliferating chondrocytes and diffuses away from its site of production to act on PTH/PTHrP receptor bearing cells (Kroneberg et al., 2006). PTHrP stimulation maintains chondrocytes in a proliferative state and prevents hypertrophy (Lee et al., 1996). Therefore, further away from the source of PTHrP production chondrocytes will undergo hypertrophy and initiate Ihh expression, which in turn will induce further PTHrP expression. This negative feedback loop is very well studied and important for normal endochondral ossification. Ihh has also been shown to be upstream of Wnt signaling and some Wnt proteins seem to modulate the effects of Ihh on chondrocytes.

Bone morphogenetic proteins (BMPs) and their inhibitors have been shown to regulate proliferation of chondrocytes in growth cartilage (Minina et al., 2001; Yoon el al., 2006). BMP-6 stimulates cartilage maturation and is important in the PTHrP/Ihh feedback cycle (Grimsrud et al., 1999). BMP-2 signaling leads to Runx2 expression and maintains mmp13 and collagen X expression in hypertrophic chondrocytes. BMP signaling is required for completion of terminal hypertrophic differentiation (Yoon et al., 2006) and is required for appropriate endochondral ossification. As with Ihh, BMP signaling can increase Wnt expression. Conversely, Wnt signaling can also increase

BMP signaling. Interestingly, BMP can also downregulate Wnt signaling via its ability to upregulate sclerostin expression, which is a Wnt inhibitor (Kamiya et al., 2008).

The fibroblast growth factor (FGF) receptor expression in chondrocytes is induced by thyroid hormone and antagonized by BMP signaling (Minina et al., 2002; Barnard et al., 2005). This receptor is expressed by proliferating and early hypertrophic chondrocytes. Stimulation by FGF leads to an inhibition of chondrocyte proliferation and acceleration of hypertrophy (Minina et al., 2002). FGF induces expression of Sox2, a member of the high mobility group domain Sox family of transcription factors. Sox2 has been shown to interfere with the transcriptional activity of β-catenin/LEF transcription leading to a downregulation of Wnt/β-catenin signaling (Mansukhani et al., 2005). FGF's effects on endochondral ossification are likely due to its downstream effects on Wnt signaling.

Vascular endothelial growth factor (VEGF) is an essential factor for angiogenesis and is expressed by hypertrophic chondrocytes in the epiphyseal growth plate (Gerber et al., 1999). In mice, inactivation of VEGF allowed for normal chondrocyte proliferation, differentiation and maturation, but resorption did not occur and bone growth (endochondral ossification) was inhibited (Gerber et al., 1999). The regulation of VEGF gene expression is complex, but Wnt signaling plays an important role. The TCF transcription factor that is controlled by β-catenin is 805 base pairs upstream of the transcriptional start site for VEGF (Zhang et al., 2001). Activation via Wnt/β-catenin signaling leads to increased gene expression of VEGF.

Platelet derived growth factor (PDGF) has been shown to be an important factor in endochondral ossification. In chondrocyte cultures, PDGF was shown to promote

proliferation and expression of matrix typical of resting zone chondrocytes and inhibit the progression of cells to a more mature growth phenotype, thus inhibiting endochondral ossification (Kieswetter etl al., 1997). Also, PDGF activation leads to downstream inactivation of glycogen synthase 3β. Inactivation of glycogen synthase 3β can lead to increased intracellular β catenin levels and indirect activation of the Wnt/β-catenin signaling pathway (Fischer et al., 2007).

Retinoic Acid Receptor signaling pathway is an important pathway in skeletogenesis and endochondral ossification. In culture, prehypertrophic or early hypertrophic chondrocytes are unable to advance in maturation unless provided with natural retinoids. Treatment with retinoids leads to upregulation and expression of type X collagen and alkaline phosphatase, both markers of terminal hypertrophy in chondrocytes (Koyama et al., 1999). Increased retinoic acid in chondrocytes increases Wnt/β- catenin signaling (Yasuhara et al., 2010) and is an important component of endochondral ossification.

As described above, many of the signaling pathways involved in endochondral ossification overlap with Wnt signaling and Wnt signaling may be the final common pathway controlling endochondral ossification (Figure 3; Milat and Ng 2009).

Wnt Signaling in endochondral ossification

Comprehensive gene expression analysis of all Wnt genes and their major secreted antagonists during mouse limb development, cartilage differentiation, and in the post-natal growth plate have shown expression of specific Wnt molecules. In the post-

natal growth plate, of the 19 known Wnt proteins, Wnt 2b, 4, 5a, 5b, 10b and 11 were all shown to be expressed suggesting their roles in postnatal endochondral bone formation (Andrade et al., 2007). Similarly, during mouse limb development, Wnt 2b, 5b, 10b, and 11 were also shown to be expressed along with Sfrp1,3,5, Dkk1, and Wif 1 (Witte et al., 2009). More specifically, Wnt 5b and Sfrp3 were both expressed in prehypertrophic and hypertrophic chondrocytes. Wnt 11 was expressed in prehypertrophic chondrocytes and throughout the neonatal growth plate, while Wnt 4 showed very strong expression throughout the growth plate. In contrast to Sfrp3, Sfrp1 and 5 were expressed in prehypertrophic and proliferating chondrocytes. Wif 1 was expressed in all chondrocytes and DKK1 was expressed in the perichondrium (Andrade et al., 2007; Witte et al., 2009). Although these genes have been shown to be expressed, the relative levels of expression have not been elucidated.

Wnt signaling has been shown to regulate chondrocyte differentiation, proliferation, maturation, and endochondral ossification (Church et al., 2002; Hu et al., 2005; Tamamura et al., 2005; Day and Yang. 2008). Activation of Wnt signaling in chondrocytes has been shown to stimulate ectopic endochondral ossification (Kitagaki et al., 2003). The activation and actions of β-catenin in endochondral ossification appears to be dependent upon the maturity of the chondrocytes. In immature chondrocytes, β-catenin suppresses hypertrophy, leading to a failure of endochondral ossification (Tamamura et al., 2005). However, in mature chondrocytes, β-catenin promotes terminal differentiation and the expression of MMP-13 and VEGF (Tamamura et al., 2005). In general, Wnt signaling appears to be predominantly downstream of Ihh signaling, but can also act upstream of Ihh signaling when inhibiting chondrocyte apoptosis (Mak et al.,

2006). Inactivation of β-catenin in differentiated chondrocytes leads to dramatically increased cell death (Mak et al., 2006). Thus it appears that Wnt signaling has multiple roles, depending on the stage of cell maturation.

Endochondral ossification is a highly complex process that requires careful regulation of chondrocyte behavior and vascular invasion. Regulation is carefully coordinated via a multitude of signaling pathways and molecules. There is considerable evidence of interactions between all the signaling pathways involved in endochondral ossification and the Wnt signaling pathway. These interactions stress the importance of Wnt signaling in endochondral ossification. Failure or inappropriate signaling associated with endochondral ossification could in theory lead failures of endochondral ossification and diseases of articular cartilage.

Osteochondrosis

Osteochondrosis (OC) is a failure of normal endochondral ossification at the articular-epiphyseal complex during the first year of life leading to retained cartilage cores which can result in fissure formation of articular cartilage along the osteochondral junction and osteochondrosis dissecans (Ytrehus et al., 2011). The disease process is thought to be multifactorial, but a definitive etiopathogenesis has eluded researchers. In some cases, there is a failure of normal chondrocyte differentiation associated with the accumulation of large numbers of small rounded chondrocytes surrounding cartilage canals leading to characteristic osteochondrosis lesions (Shingleton et al., 1997).

Osteochondrosis has been shown to be associated with rapid growth rate (van Weeren et al., 1999), biomechanical trauma (van Weeren and Barneveld, 1999), altered blood supply and chondronecosis (Carlson et al., 1995; Olstad et al,, 2011) and molecular aberrations (Semevolos et al., 2001, 2002, 2005; Nixon et al., 2008; Mirams et al., 2009; Riddick et al., 2013). It has been shown to be heritable (Dierks et al., 2007; Wittwer et al., 2007; Gevenhof et al., 2009) and is of major concern to a multitude of species including humans, dogs, pigs, and equids. There has been an enormous amount of research into the pathogenesis of osteochondrosis in horses due to its role in lameness in young horses and the monetary losses associated with the disease.

Cartilage canals have been implicated as a factor in the etiopathogenesis of osteochondrosis (Carlson et al., 1995; Shingleton et al., 1997; Olstad et al., 2008). Articular cartilage is avascular throughout life and derives most of its nutrition from synovial fluid. The articular-epiphyseal complex is too thick to receive nutrition from synovial fluid and is dependent on a patent vascular supply in the form of cartilage canals in order to survive and undergo appropriate endochondral ossification (Figure 1; Hodge and McKibbin, 1969). Patent cartilage canals are only present in the epiphyseal cartilage of young horses for the first few months of life and are no longer present by 7 months (Carlson et al., 1995). It has been suggested that trauma to or focal failure of cartilage canals to provide adequate blood supply to the articular-epiphyseal cartilage complex may lead to an ischemic necrosis of growth cartilage. This ischemic cartilage does not undergo normal bony conversion and represents a focal disturbance in endochondral ossification that could lead to OC (Carlson et al., 1995). Cartilage canals traverse the osteochondral junction which may predispose these small vessels to injury. In contrast to

these studies, cartilage canals of foals with early osteochondrosis have been shown to contain viable blood vessels retained below the ossification front surrounded by small, non-hypertrophic chondrocytes suggesting that patent blood vessels within the cartilage canals may allow for continued exposure of these chondrocytes to systemic hormones preventing appropriate chondrocyte maturation (Shingleton et al., 1997). Either way, cartilage canals and the chondrocytes surrounding them appear to be factors in the pathogenesis of OC in foals.

Because osteochondrosis appears to be a developmental disease process, altered autocrine and paracrine signaling factors required for endochondral ossification are believed to be a key factor involved in the pathogenesis of osteochondrosis. Previous studies in horses have looked closely at Ihh and PTHrP because, as previously described, these molecules regulate the progression of chondrocytic terminal differentiation in physeal cartilage (Vortkamp et al., 1996; Kronenberg, 2003). Dysregulation of the Ihh/PTHrp pathway has been found in advanced OC lesions (Semevolos et al., 2002, 2005; Serteyn et al., 2010) and in early OC (Riddick et al., 2012). Also, the gene expression of VEGF and MMP-13, important factors required for vascular invasion and appropriate endochondral ossification, has been shown to be increased in advanced OC lesions (Garvican et al., 2008; Nixon et al., 2008) and MMP 13 gene expression has been shown to be increased in chondrocytes surrounding cartilage canals and whole cartilage in foals with early OC (Riddick et al., 2012). Wnt signaling is down stream of Ihh and has been shown to increase expression of MMP13 and VEGF (Tamamura et al., 2005, Reis and Liebner 2013). Based these findings, Wnt signaling is positioned to be a major factor in the pathogenesis of OC in horses.

Previous gene expression profiling from leukocytes have shown aberrant gene expression of components of the canonical and non-canonical Wnt signaling and retinoic acid pathways in horses afflicted with osteochondrosis (Bertone and Reed, 2008; Serteyn et al., 2010). In addition, evaluation of differential gene expression in radiographically evident (OC manifesta) lesions in 7-9 month old horses indicated that Wnt/β-catenin pathways are altered in horses with advanced OC (Nixon et al., 2008). The objective of this study was to elucidate the expression of signaling molecules associated with the Wnt signaling pathway in cartilage of prepubescent foals and determine their association with chondrocytes located at the osteochondral junction and cartilage canals. Our hypothesis was that increased expression of the components of the Wnt signaling pathway in chondrocytes of the osteochondral junction and cartilage canals would be found in early osteochondrosis (OC) lesions when compared to normal controls

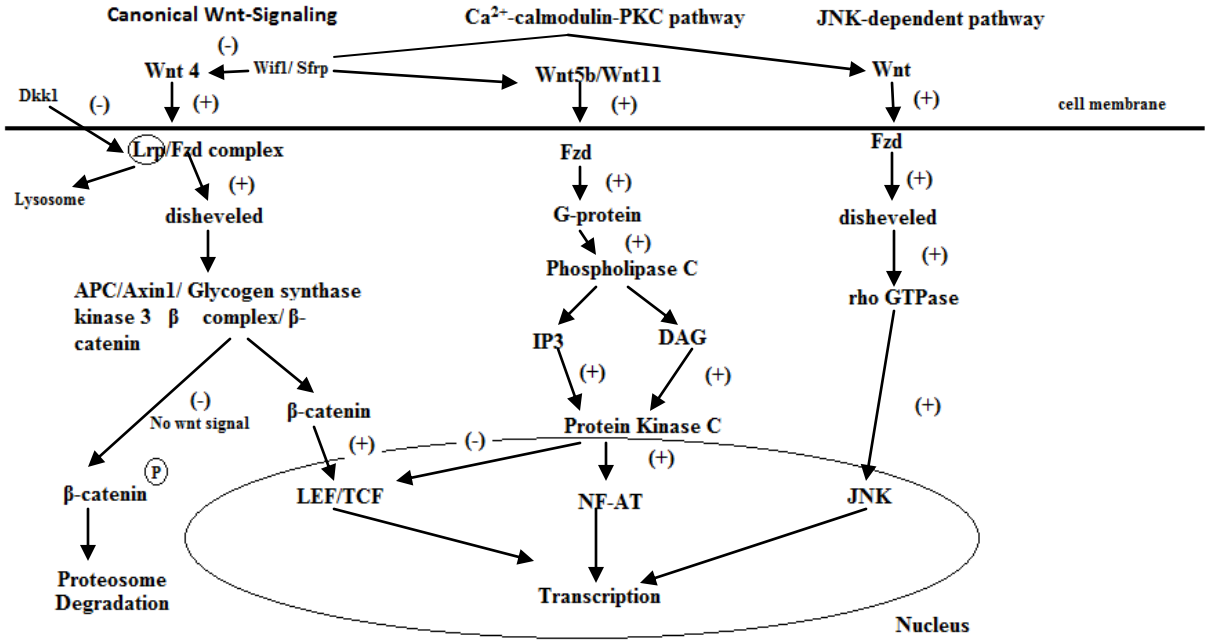

Figure 1. Schematic description of Wnt pathways. General overview of canonical and non-canonical Wnt pathways as discussed in text. (+) represent activation, (-) inhibition.

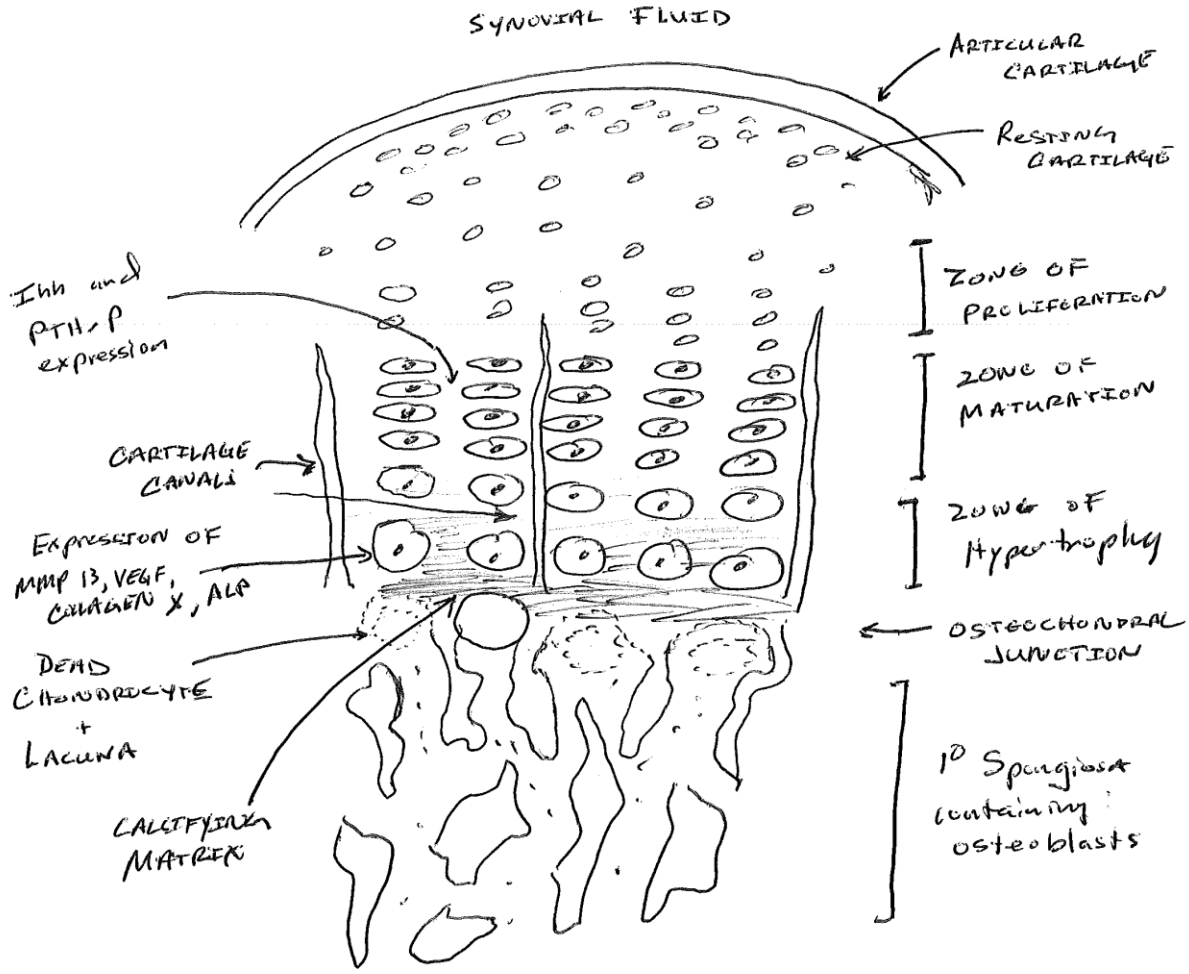

Figure 2. Diagram of normal endochondral ossification. Resting chondrocytes become proliferative, mature, and hypertrophy. Hypertrophied chondrocytes undergo cell death and the lacuna left behind are invaded by and filled with bone

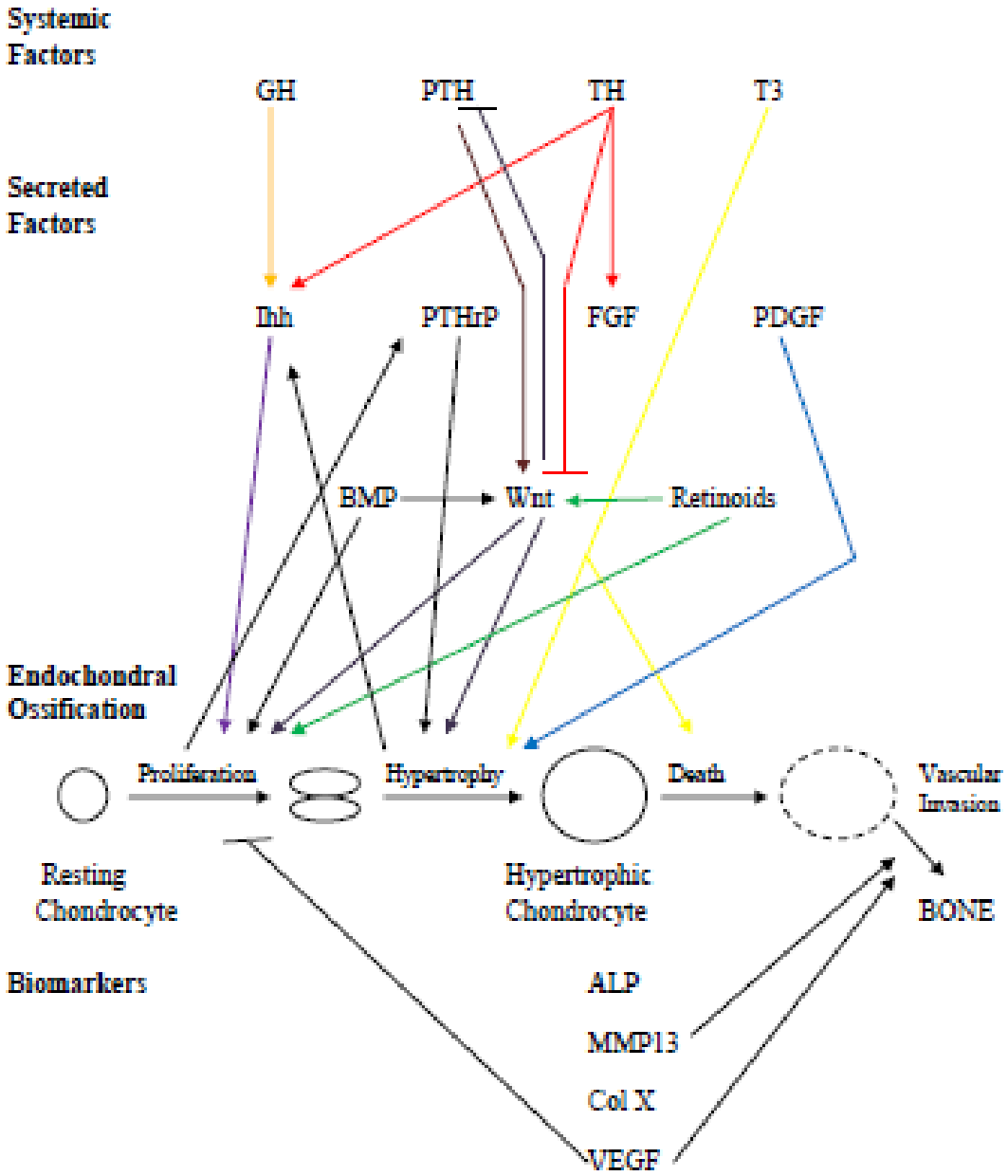

Figure 3. Summary of possible effects of signaling molecules at different phases during endochondral ossification. Lines with arrows indicate a positive effect. Lines with a flat line at the end indicate an inhibitor effect.

Chapter 2

Materials and Methods

Sample Collection— Archived osteochondral and cartilage samples have been obtained from the femoropatellar joints of immature horses from 1-6 months of age, yielding 6 foals with early OC and 8 normal controls (Riddick et al. 2012). Institutional Animal Care and Use committee approval was obtained prior to start of this project. Foals were euthanatized for reasons unrelated to lameness or joint sepsis (sodium pentobarbital, 105 to 187 mg/kg IV). Osteochondral samples were sharply dissected from the lateral and medial trochlear ridges of both distal femurs. Full thickness cartilage samples from both trochlear ridges immediately adjacent to the osteochondral sampling sites were then sharply dissected, snap frozen in liquid nitrogen, and stored at -80°C prior to RNA isolation. Osteochondral samples were either snap frozen in OCT medium[a] and stored at -80^{o}C, or fixed in 4% paraformaldehyde for 48 hours and transferred to 10% EDTA solution for decalcification (2-4 weeks). Decalcified samples were embedded in paraffin and sectioned for immunohistochemistry[b] and H&E staining.

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 early OC, advanced OC, or normal. Normal cartilage was defined as

having no gross or histologic abnormalities. Early OC was defined as samples having altered endochondral ossification (locally thickened cartilage, loss of normal columnar arrangement of chondrocytes, chondrones) (class 1 and 2) or separation (fissures, necrosis) along the osteochondral junction without concurrent superficial cartilage lesions (class 3) (van Weeren and Barneveld 1999). Advanced OC was defined as having separation (fissures, necrosis) along the osteochondral junction with concurrent superficial lesions (OC flaps, osteochondral fragments, fibrocartilage formation) (class 4) (van Weeren and Barneveld 1999). Based on these criteria, 6 foals were determined to have early OC, and 8 were classified as normal (Riddick et al., 2012). In OC samples, 6 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 1).

Laser-capture microdissection—Frozen osteochondral samples were sectioned using a cryomicrotome and mounted on slides using a tape transfer system[c] and stored at -80^{o}C. Immediately prior to laser capture microdisection (LCM), each slide was dehydrated in graded alcohol and xylene. LCM was performed (Riddick et al., 2012) using PIXCELL II Laser Capture Microdissection System[d] and CapSure Macro LCM caps[e]. Chondrocytes were captured immediately surrounding the cartilage canals and osteochondral junction (separate sites) of each animal. Up to 8 caps from sequential sections were combined for each site (approximately 400-800 cells per site) (Figure 4).

RNA Isolation— The PicoPure RNA Isolation Kit[d] was used for RNA isolation of LCM samples with slight modifications. Briefly, cell lysate from up to 8 caps was loaded onto

a pre-conditioned RNA-purification column[g]. The column was then washed and treated with RNase-free DNase prior to RNA elution from the column. RNA quality control was performed using an Agilent 2100 Bioanalyzer[f] and the RNA 6000 Pico LabChip kit[f]. Full thickness articular cartilage specimens for total RNA isolation were pulverized in a freezer mill[j] prior to guanidine isothiocyanate denaturation[k], chloroform extraction, isopropanol precipitation, and RNA purification[g]. Purity and concentration were assessed by agarose gel electrophoresis and UV spectrophotometry[l].

Real-time quantitative RT-PCR— Two-step quantitative real time RT-PCR was performed as described in the manufacturer's protocol[e] to evaluate expression of equine-specific β-catenin, Wnt-4, Wnt-5b, Wnt-11, Dickkopf-1 (Dkk-1), low density lipoprotein receptor-related protein (Lrp4, Lrp6), Axin1, Wnt inhibitory factor (WIF)-1, secreted Frizzled-related protein 1 (Sfrp1), Sfrp3, Sfrp5, retinoic acid receptor gamma (RARgamma), RAR-inducible serine carboxypeptidase (SC-PEP) and 18S mRNA expression levels mRNA, using a real-time PCR system[h]. First strand cDNA synthesis was accomplished with reverse transcription, random hexamers, RNA from whole cartilage samples and RNA from the laser captured samples. Logarithmic preamplification (11 cycles), using pooled equine specific primer pairs (β-catenin, Wnt 4, Wnt 5b, Wnt 11, Wif 1, Sfrp1, Sfrp3, Sfrp5, Lrp4, Lrp6, Axin1, Dkk1, RARγ, SC-PEP) and Taqman PreAmp Master Mix kit, was performed on cDNA samples. 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 standard curve. PCR was performed in duplicate using 20ul

final reaction mixture of 2X Taqman® Gene Expression Master Mix[e], 250nM probe, 900nM forward and reverse primers, and 7.5ul pre-amplified sample cDNA and 18S RNA was used as the housekeeping gene for normalizing gene expression. After a two minute incubation at 50°C activationg uracil-DNA glycosylase (UDG) and ten minute incubation at 95°C to deactivate UDG and activate AmpliTaq®Gold DNA polymerase, 40 PCR cycles of fifteen seconds of 95°C followed by 1 minute of 60°C were run.

Western Blot— Protein was extracted from whole cell equine renal cells and equine liver using Radio-Immunoprecipitation assay (RIPA) extraction buffer[o] and sonication. Protein levels were determined via a Bradford assay using a known concentration of bovine serum albumin for the standard curve. The protein sample was mixed with 4x buffer with mercaptoethanol and placed in boiling water for 60 seconds. The wells of a NuPage® 4-12% Bis-Tris gel[m] with 10X1.0mm wells were loaded with 40ug of protein/well with one well containing 10ul of BenchMark®Pre-Stained Protein Ladder[m]. The gels were loaded into an XCell SureLock®Electrophoresis[m] cell with NuPage® MES SDS running buffer[m]. The gels were run at 200V for 45-60 minutes. At completion, the running buffer was removed and nitrocellulose membranes and gels were prepared for transfer using NuPage® Transfer Buffer[m] in the same XCell SureLock® Electrophoresis cell[m]. The system was run at 30-34V for one hour. Following transfer, the membranes were blocked with bovine serum albumin (BSA) for 45 minutes at room temperature. The primary antibody was diluted 200:1 and applied to the membranes for 12 hours at 4°C. The non-specific primary antibody binding was washed with three 5-minute washes. The secondary antibody with linked horseradish peroxidase[p] was applied

in a 1:25,000 dilution at room temperature for 45 minutes. Super Signal® West Pico Chemiluminescent Substrate[n] was applied and x-ray film used to detect specific protein binding. Equine protein binding could only be confirmed for rabbit α-human polyclonal[p] (β catenin, Wnt 11) and mouse α-human monoclonal[q] (DKK1, Wnt 4) (Figure 5).

Immunohistochemistry—Immunohistochemistry was performed on 6μm osteochondral sections using 1:20 dilution of rabbit α-human polyclonal[q] (β catenin, Wnt 11) or mouse α-human monoclonal[q] (DKK1, Wnt 4) primary antibodies and the Supersensitive Link-label Multilink Immunohistochemistry System[i], as described previously (Semevolos et al., 2006). Negative procedural controls were confirmed by using non-immune serum in place of primary antibody. Breifly, the cartilage sections were deparaffinized and brought to water then incubated at 37°C for 60 minutes under a testicular hyaluronidase solution to expose the antigen. Endogenous peroxidases were quenched via hydrogen peroxide. Depending on the primary antibody, the appropriate non-immune serum was applied for 30 minutes, rinsed with phosphate buffer solution (PBS) (0.133M NaCl, 0.0086M K_2HPO_4, and 0.0015 M KH_2PO_4), and the appropriate primary antibody applied for 60 minutes at room temperature in a humid chamber. The samples were again rinsed with PBS and the biotinylated secondary multilink antibodies were applied. The samples were then labeled with streptavidin conjugated peroxidase and the production of a color product was induced via application of diaminobenzidine tetrachloride (DAB). The sections were then counterstained with Harris hematoxylin and coverslipped in preparation for microscopic evaluation. Negative procedural controls were confirmed by using non-immune serum in place of primary antibody.

Immunohistochemistry scoring— Immunohistochemistry samples were evaluated by 2 investigators (SAS and MAK), who were blinded to group allocation of samples. Protein expression was scored from 0 to 3: 0 (no staining/expression), 1 (mild staining/expression), 2 (moderate staining/expression), or 3 (strong staining/expression). First, specific cell populations were scored, including: 1) chondrocytes surrounding the cartilage canals and 2) chondrocytes adjacent to the osteochondral junction. The scores of the two observers were averaged for combined scores at each location. Second, each of three cartilage layers (superficial, middle, deep) was scored and the scores of each layer were added together for a total cartilage score which was then averaged between the two observers (Figure 6).

Statistical Analysis--Immunohistochemistry scores and quantitative comparisons from real-time PCR assays were compared between OC and normal horses using a Wilcoxon rank sum test and Mann Whitney U test ($P<0.05$).

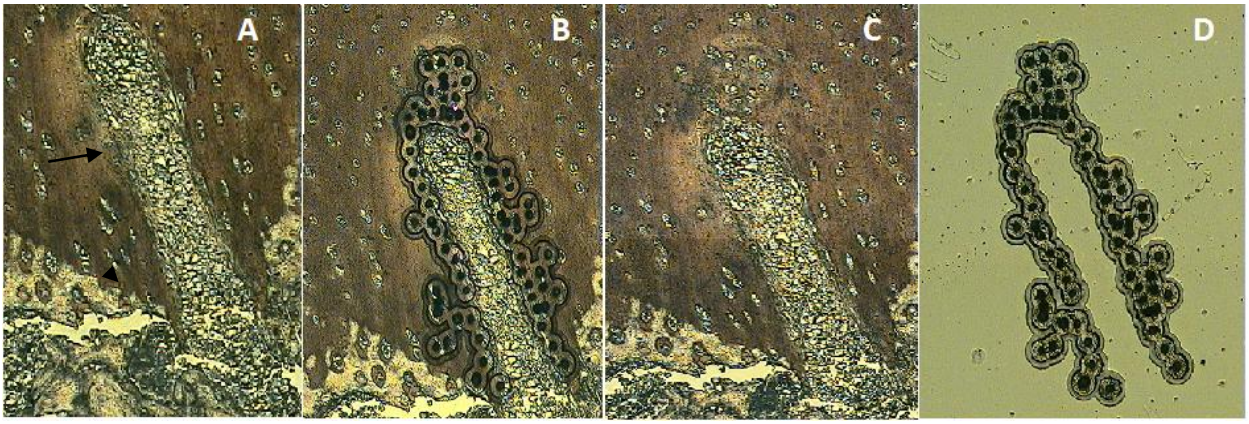

Figure 4. Photomicrographs of the laser capture technique from a 4-month-old foal. (A)Prelaser image showing a cartilage canal (arrow) at the osteochondral junction (arrowhead). (B) Post laser showing the specific chondrocytes captured (C) Post removal of the cap with the missing chondrocytes (D) LCM cap post removal containing the chondrocytes of interest.

Band Number	Average Molecular Weight (kDa)
1	~180
2	~115
3	~82
4	~64
5	~49
6	~37
7	~26
8	~19
9	~15
10	~6

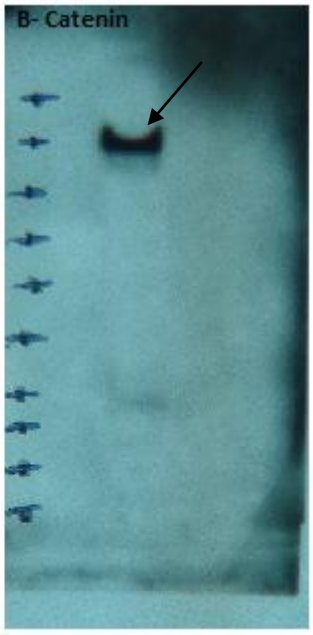

Figure 5. Radiograph of chemiluminescent western blot analysis to confirm specific binding of rabbit α-human polyclonal[q] β catenin to equine β catenin (arrow). The key for the BenchMark®Pre-Stained Protein Ladder[m] is on the left and the molecular weight of the protein that the rabbit α-human polyclonal β catenin is shown. The band is between 115 and 82 kDa, with β catenin reported to have a molecular weight of around 86 kDa.

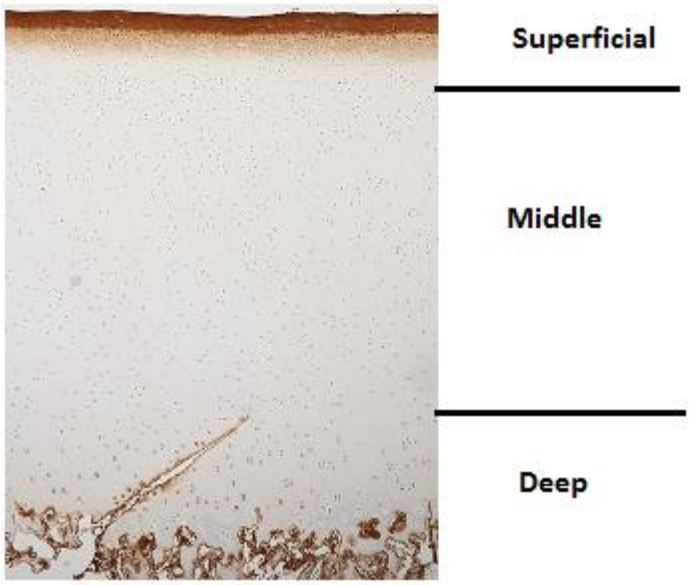

Figure 6. Photomicrographs of osteochondral sections following immunohistochemical localization showing the different cartilage layers scored by each investigator.

Table 1. Summary of animals and samples from the study with descriptions of the gross and histologic abnormalites. OCD- osteochondritis dissecans; LCM- laser capture microdissection; CC- cartilage canal; OCJ- osteochondral junction C-colt; F-filly; Y-test performed; N-test not performed; NLS-no lesions seen

Foal	**Classification**	**Age-mo.**	**Sex**	**Description**	**LCM-CC**	**LCM-OCJ**	**IHC**
1	OCD	5	C	Cartilage easily separated from subchondral bone, chondronecrosis along osteochondral junction	N	N	Y
2	OCD	4	C	Thickened cartilage easily separated from subchondral bone	Y	Y	Y
3	OCD	5	C	Thickened cartilage easily separated from subchondral bone	Y	Y	Y
4	OCD	4	F	Cartilage easily separated from subchondral bone, subchondral bone hemorrhage	N	N	Y
5	OCD	1	F	Thick cartilage with osteochondral junction containing fingerlike projections of cartilage	Y	Y	Y
6	OCD	5	F	Thin cartilage distal medial trochlear ridge, delayed ossification with extremely thickened cartilage proximally, cartilage easily separated from subchondral bone, chondronecrosis along osteochondral junction	Y	Y	Y
7	Normal	4	C	NLS	N	N	Y
8	Normal	4	F	NLS	N	Y	Y
9	Normal	4	F	NLS	Y	Y	Y
10	Normal	5	F	NLS	Y	Y	Y
11	Normal	4	F	NLS	Y	Y	Y
12	Normal	4	F	NLS	Y	Y	Y
13	Normal	6	F	NLS	N	N	Y
14	Normal	3	F	NLS	Y	Y	Y

CHAPTER 3

Results

In laser captured chondrocytes along the osteochondral junction (Figure 7; Table 2) gene expression was significantly increased for β-catenin (p=0.004) and SC-PEP (p=0.02) in foals with OC lesions compared to normal controls. In chondrocytes adjacent to cartilage canals (Figure 8; Table 3), there was significantly increased gene expression of β-catenin (p=0.02), Wnt -5b (p=0.04), WIF-1 (p=0.02), Lrp6 (p=0.02), Dkk-1 (p=0.01), Axin1 (0.04), and SC-PEP (p=0.02), as well as a significantly decreased gene expression of Wnt-11 (p=0.04) in OC foals vs. normal controls. No difference in gene expression was found for Sfrp1, Sfrp3, Sfrp5, and Lrp4 in the laser captured samples between OC and normal cartilage samples.

In full thickness articular cartilage samples, there was no significant differences in gene expression of β-catenin, Wnt 4, Wnt 11, Wif 1, LRP6, Dkk1, Axin1, SC-PEP, or RARγ in OC cartilage when compared to normal controls (Figure 9; Table 4).

Immunostaining for β-catenin was mild in the superficial and middle cartilage layers and moderate in the deep cartilage layer, including chondrocytes along the osteochondral junction. There was mild β-catenin protein expression in chondrocytes surrounding cartilage canals (Figure 10). No significant difference was found in cartilage canal, osteochondral junction, or total cartilage scores between OC and normal samples (Tables 5, 6 and 7)

Strong Wnt-11 protein expression was apparent in the superficial cartilage layers and vascular cells lining the cartilage canals and osteochondral junction, with moderate protein expression in chondrocytes surrounding cartilage canals (Figure 11). Protein

expression of Wnt 11 was significantly decreased (p=0.02) in the superficial cartilage layer and a trend (p=0.08) for increased protein expression in the deep layer when compared to normal controls. There was no significant difference in cartilage canal, osteochondral junction, or total cartilage scores between OC and normal samples (Tables 5, 6 and 7).

Wnt 4 protein expression was most apparent along the osteochondral junction and the deep cartilage layer with mild protein expression in chondrocytes adjacent to the cartilage canals and in the middle and superficial layers (Figure 12). There was no significant difference in immunostaining in cartilage canal, osteochondral junction, or total cartilage scores between OC and normal samples (Tables 5, 6 and 7).

Protein expression of Dkk1 was strongest along the osteochondral junction. Mild protein expression was present in the superficial, middle, and deep layers as well as in chondrocytes adjacent to cartilage canals (Figure 13). Protein expression of Dkk1 was significantly decreased (p=0.02) in the deep cartilage layer when compared to normal controls. There was no significant difference in immunostaining in cartilage canal, osteochondral junction, or total cartilage scores between OC and normal controls (Tables 5, 6 and 7).

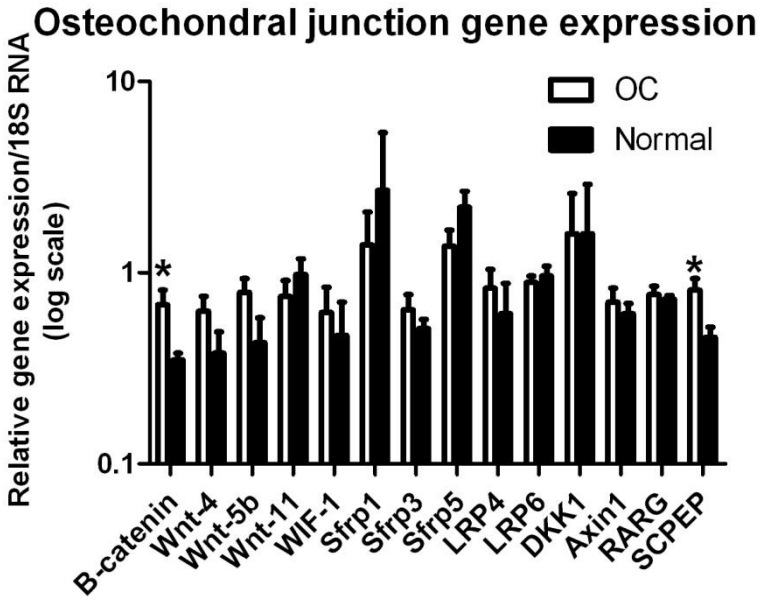

Figure 7. Gene expression of laser-captured osteochondral junction chondrocytes using RT-PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18s RNA. Significantly increased gene expression is represented with a *

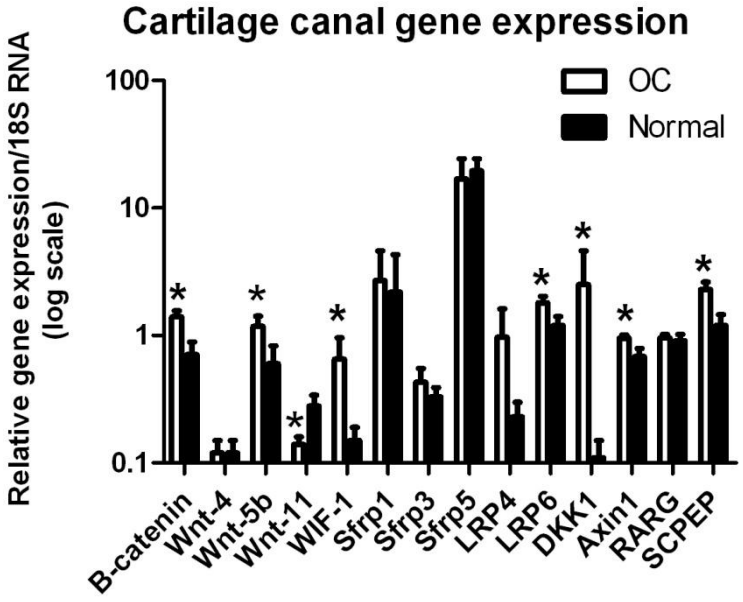

Figure 8. Gene expression of laser-captured cartilage canal chondrocytes using RT-PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18s RNA. Significantly increased gene expression is represented with a *

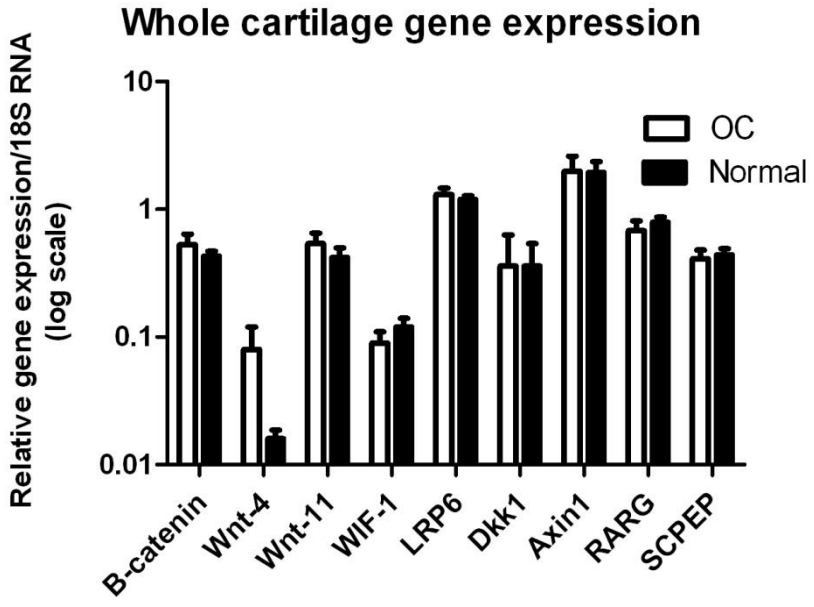

Figure 9. Gene expression of full thickness articular cartilage samples using RT-PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18s RNA. No significant differences were appreciated.

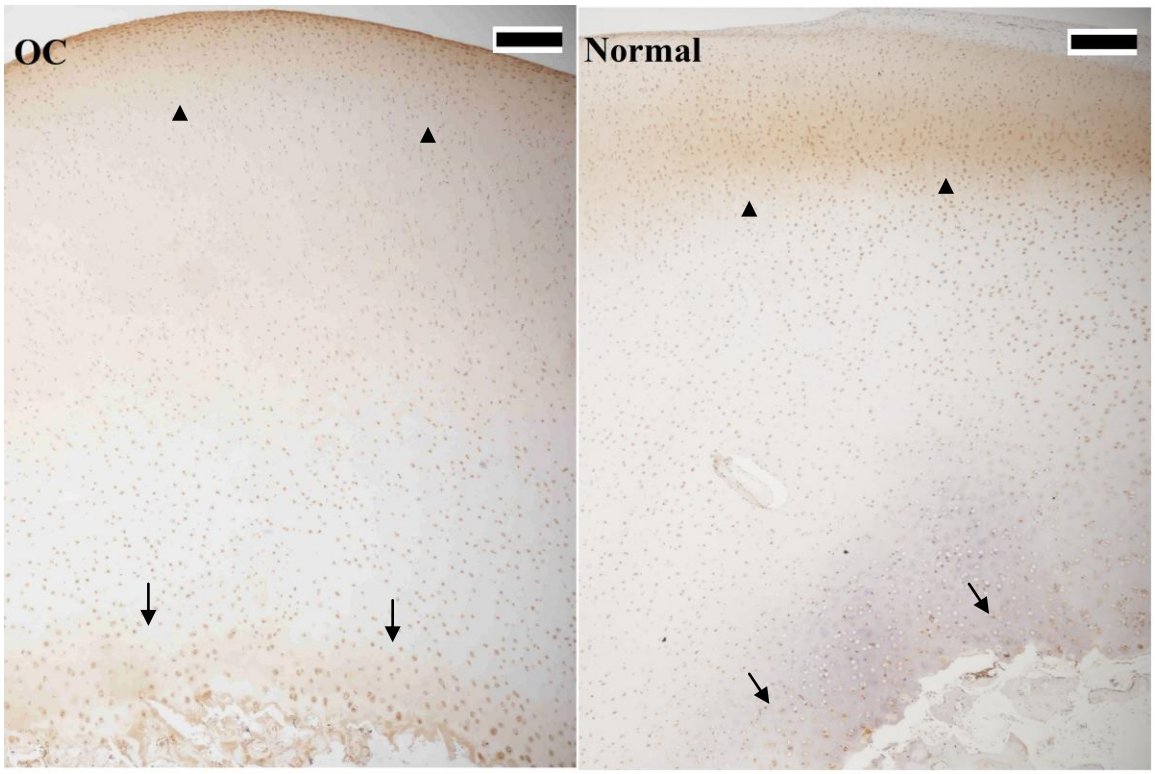

Figure 10. Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against β-catenin. Similar expression pattern can be seen between a 5-month-old colt withosteochondrosis (OC) and a normal 4-month-old filly with the strongest protein expression in the superficial cartilage layer (arrowheads) with significant protein expression in the deep cartilage layer and along the osteochondral junction (arrows). Magnification at 40X. The black bar represents 200um.

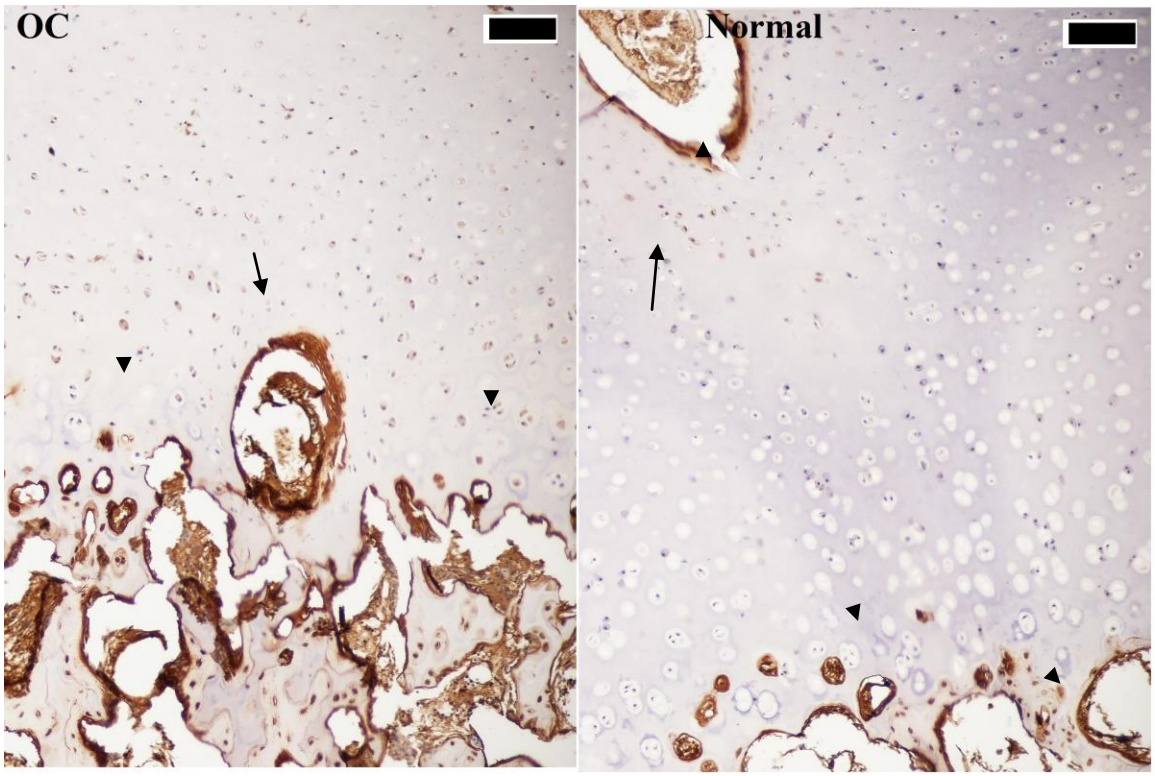

Figure 11. Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against Wnt 11. These sections are focused in on the deep cartilage layer and the osteohondral junction. Similar expression pattern can be seen between a 4-month-old colt withosteochondrosis (OC) and a normal 4-month-old filly with the strongest expression in the endothelial cells surrounding cartilage canals and osteochondral junction (arrowheads) with some staining of chondrocytes around cartilage canals (arrows). Maginification at 100X. The black bar represents 100um.

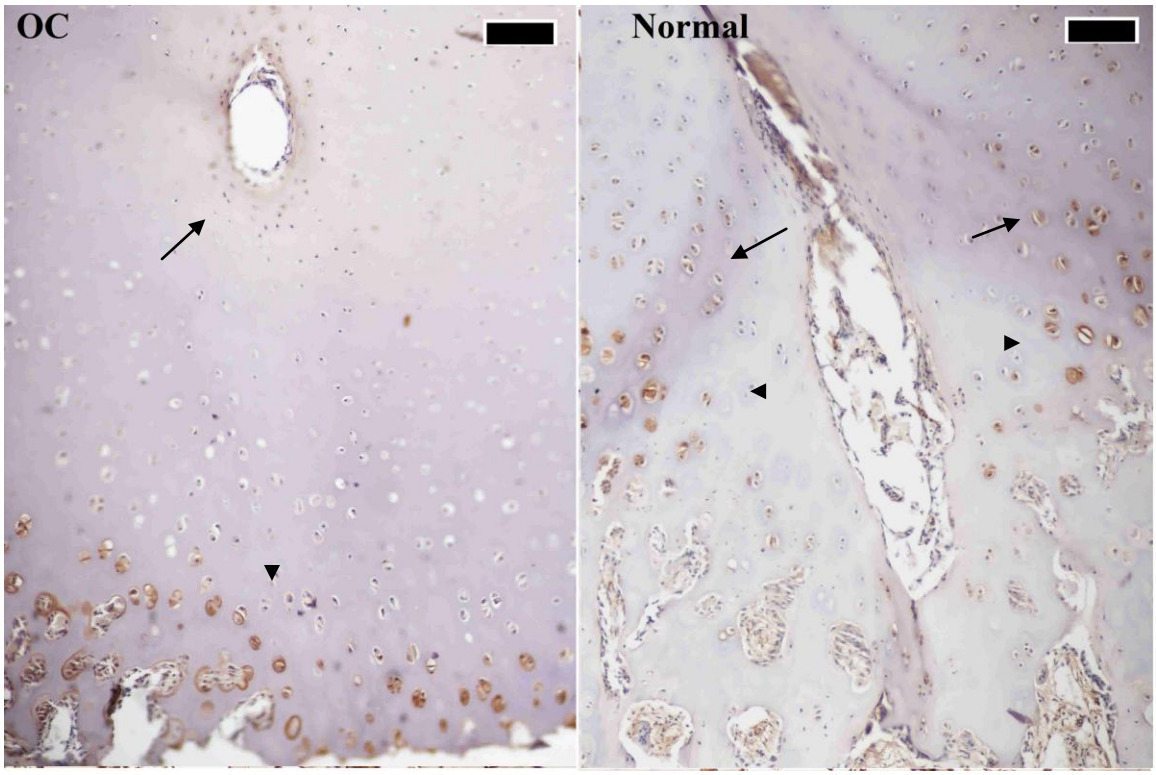

Figure 12. Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against Wnt 4. These sections are focused in on the deep cartilage layer and the osteohondral junction. Similar expression pattern can be seen between a 5-month-old colt with osteochondrosis (OC) and a normal 4-month-old filly with the strongest expression at the osteochondral junction(arrow heads) and around the cartilage canals(arrows). Maginification at 100X. The black bar represents 100um.

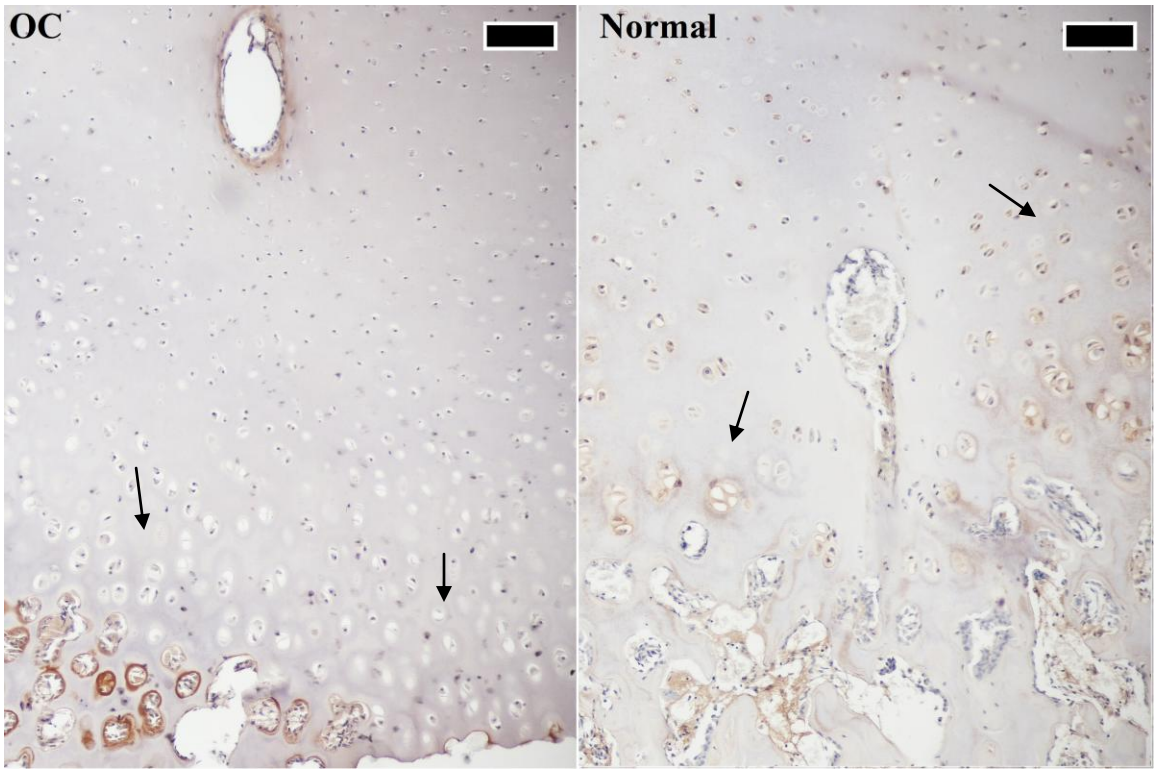

Figure 13. Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against Dkk1. These sections are focused in on the deep cartilage layer and the osteohondral junction. Similar expression pattern can be seen between a 5-month-old colt with osteochondrosis (OC)l and a normal 3-month-old filly with mild expression in the layers shown with a significantly decreased expression in the deep layer (arrows) of osteochondrosis foals vs normal controls. Maginification at 100X. The black bar represents 100um.

Table 2. Mean gene expression +/- SEM quantified by real-time PCR in laser captured osteochondral junction chondrocytes from early OC and normal control horses. Relative gene expression was normalized by dividing the target gene by 18S expression.

Target gene	**Early OC**	**Normal Control**	***P*-value**
B-catenin	0.68 +/- 0.13	0.35 +/- 0.03	0.004
Wnt 4	0.63 +/- 0.12	0.39 +/- 0.12	0.06
Wnt 5b	0.79 +/- 0.14	0.43 +/- 0.15	0.06
Wnt 11	0.75 +/- 0.16	0.98 +/- 0.21	0.22
Wif 1	0.62 +/- 0.22	0.47 +/- 0.23	0.44
Sfrp 1	1.35 +/- 0.68	2.72 +/- 2.72	0.22
Sfrp 3	0.64 +/- 0.13	0.51 +/- 0.06	0.36
Sfrp 5	1.38 +/- 0.29	2.21 +/- 0.47	0.12
Lrp 4	0.83 +/- 0.21	0.61 +/- 0.27	0.17
Lrp 6	0.89 +/- 0.07	0.96 +/- 0.12	0.63
Axin 1	0.70 +/- 0.13	0.61 +/- 0.08	0.44
Dkk 1	1.63 +/- 1.00	1.61 +/- 1.36	0.12
RAR γ	0.77 +/- 0.08	0.73 +/- 0.03	0.36
SC-PEP	0.81 +/- 0.12	0.46 +/- 0.06	0.02

Table 3. Mean gene expression +/- SEM quantified by real-time RT-PCR in laser captured cartilage canal chondrocytes from early OC and normal control horses. Relative gene expression was normalized by dividing the target gene by 18S expression.

Target gene	**Early OC**	**Normal Control**	***P*-value**
B-catenin	1.40 +/- 0.16	0.72 +/- 0.18	0.02
Wnt 4	0.12 +/- 0.03	0.12 +/- 0.03	0.43
Wnt 5b	1.18 +/- 0.24	0.06 +/- 0.23	0.04
Wnt 11	0.14 +/- 0.02	0.28 +/- 0.06	0.04
Wif 1	0.65 +/- 0.31	0.15 +/- 0.04	0.02
Sfrp 1	2.73 +/- 1.93	2.23 +/- 2.08	0.53
Sfrp 3	0.43 +/- 0.12	0.33 +/- 0.06	0.28
Sfrp 5	16.98 +/- 7.43	19.50 +/- 4.89	0.36
Lrp 4	0.97 +/- 0.65	0.23 +/- 0.07	0.12
Lrp 6	1.80 +/- 0.23	1.24 +/- 0.21	0.02
Axin 1	0.95 +/- 0.06	0.68 +/- 0.11	0.04
Dkk 1	2.55 +/- 2.07	0.12 +/- 0.04	0.01
RAR γ	0.96 +/- 0.06	0.91 +/- 0.11	0.22
SC-PEP	2.27 +/- 0.32	1.25 +/- 0.26	0.02

Table 4. Mean gene expression +/- SEM quantified by real-time RT-PCR in full thickness articular cartilage from early OC and normal control horses. Relative gene expression was normalized by dividing the target gene by 18S expression.

Target gene	**Early OC**	**Normal Control**	***P*-value**
B-catenin	0.53 +/- 0.11	0.43 +/- 0.04	0.31
Wnt 4	0.08 +/- 0.04	0.02 +/- 0.002	0.08
Wnt 11	0.54 +/- 0.11	0.42 +/- 0.08	0.31
Wif 1	0.09 +/- 0.02	0.12 +/- 0.02	0.36
Lrp 6	1.30 +/- 0.16	1.21 +/- 0.08	0.36
Axin 1	1.99 +/- 0.62	1.94 +/- 0.42	0.42
Dkk 1	0.36 +/- 0.27	0.37 +/- 0.18	0.42
RAR γ	0.68 +/- 0.13	0.80 +/- 0.08	0.09
SC-PEP	0.41 +/- 0.07	0.43 +/- 0.05	0.55

Table 5. Mean protein expression scores +/- SEM following immunohistochemistry of osteochondral sections from early OC and normal controls. Each component was scored from 0 (no staining/expression) to 3 (strong staining/expression), and the scores of the two observers (SAS and MAK) were averaged.

Target protein	**Site**	**Early OC**	**Normal Control**	***P*-value**
β-catenin	Cartilage Canal	0.75 +/- 0.27	1.05 +/- 0.35	0.36
	Osteochondral Junction	2.18 +/- 0.13	2.11 +/- 0.13	1.0
Wnt 4	Cartilage Canal	0.54 +/- 0.36	0.25 +/- 0.25	0.45
	Osteochondral Junction	1.65 +/- 0.32	1.56 +/- 0.25	0.86
Wnt 11	Cartilage Canal	1.25 +/- 0.24	1.17 +/- 0.41	0.68
	Osteochondral Junction	1.61 +/- 0.20	1.19 +/- 0.08	0.09
Dkk1	Cartilage Canal	0.75 +/- 0.46	0.56 +/- 0.33	0.47
	Osteochondral Junction	1.58 +/- 0.36	0.92 +/- 0.29	0.12

Table 6. Mean protein expression scores +/- SEM following immunohistochemistry of osteochondral sections from early OC and normal controls. Each component was scored from 0 (no staining/expression) to 3 (strong staining/expression), and the scores of the two observers (SAS and MAK) were averaged.

Target protein	**Site**	**Early OC**	**Normal Control**	***P*-value**
β-catenin	Superficial	0.63 +/- 0.18	0.78 +/- 0.14	0.56
	Middle	0.72 +/- 0.16	1.11 +/- 0.10	0.08
	Deep	1.64 +/- 0.15	1.72 +/- 0.17	0.71
	Mean Total	3.00 +/- 0.34	3.61 +/- 0.24	0.19
Wnt 4	Superficial	0.52 +/- 0.21	0.06 +/- 0.04	0.16
	Middle	0.45 +/- 0.11	0.75 +/- 0.18	0.16
	Deep	1.20 +/- 0.20	1.14 +/- 0.16	0.71
	Mean Total	2.18 +/- 0.46	1.94 +/- 0.29	0.87
Wnt 11	Superficial	2.14 +/- 0.28	2.86 +/- 0.07	0.02
	Middle	0.36 +/- 0.15	0.17 +/- 0.08	0.44
	Deep	0.91 +/- 0.23	0.36 +/- 0.11	0.08
	Mean Total	3.41 +/- 0.48	3.39 +/- 0.22	0.95
Dkk1	Superficial	1.28 +/- 0.25	1.00 +/- 0.27	0.17
	Middle	0.20 +/- 0.08	0.25 +/- 0.07	0.42
	Deep	0.60 +/- 0.24	1.08 +/- 0.16	0.02
	Mean Total	2.08 +/- 0.39	2.33 +/- 0.35	0.54

CHAPTER 4

Discussion

To the author's knowledge, this is the first study to look at chondrocyte Wnt gene expression in foals with early osteochondrosis lesions. Previous studies of osteochondrosis have shown possible involvement of the Wnt and retinoic acid pathways in yearlings with osteochondrosis (Nixon 2008; Bertone and Reed 2008). In older horses, it is difficult to determine if increases in gene expression are due to osteochondrosis or due to a secondary inflammatory response to the lesion. Our study has two major advantages when compared to previous OC studies in that: (1) our samples are from early OC before the onset of inflammatory changes and (2) through the process of laser capture microdissection we are able to look at two very distinct populations of chondrocytes hypothesized to be involved in the etiopathogenesis of OC. Laser capture microdissection has been shown to increase sensitivity and specificity for the detection of genes involved in disease processes (Szaniszlo et al., 2004). Osteochondrosis is a disease process that involves specific areas of developing cartilage and possibly specific chondrocyte populations. If this chondrocyte population is a minor cell subset of the total chondrocytes tested in whole cartilage samples, then major changes in gene expression may not be great enough to be detected amongst the greater unchanged gene expression of the normal population. This concept is supported nicely by our data. The whole cartilage gene expression data was not significantly different between OC and normal foal cartilage samples, but the laser captured chondrocytes, especially those around the cartilage canals, showed statistically different expression levels that would not have been elucidated without the laser capture microdissection technology.

Wnt signaling is critical to bone formation and studies have shown the expression of Wnt 4, Wnt 5b, Wnt 11, Dkk1, and Wif 1 in developing and post natal mouse growth plates (Andrade et al., 2007, Witte et al., 2009). Activation of β-catenin LEF/TCF signaling in chondrocytes accelerates ectopic bone formation and inactivation suppresses it (Kitagaki et al., 2003). LEF/TCF expression stimulates the expression of mature chondrocyte hypertrophic traits including upregulation of MMP-13 (Kitagaki et al., 2003), which is necessary for appropriate endochondral ossification and alkaline phosphatase gene expression (Milat and Ng 2009). In MMP-13 null mice, chondrocytes undergo normal hypertrophy, but the zone of hypertrophic chondrocytes is expanded, and invasion of the ossification front is delayed in its absence (Inada et al., 2004; Stickens et al., 2004). Wnt regulation of chondrocyte differentiation is a balance of positive and negative influences to the pathway (Yates et al. 2005), and in theory, any disruption in this regulation could lead the failure of chondrocyte maturation and OC.

β-catenin gene expression was significantly elevated in the chondrocytes surrounding cartilage canals and chondrocytes along the osteochondral junction. β-catenin is tightly regulated and when Wnt signaling is absent, β-catenin is degraded and not available to bind LEF/TCF and induce expression of Wnt target genes (Milat and Ng, 2009). Continuous expression of β- catenin in mature chondrocytes stimulated hypertrophy, matrix mineralization, and expression of terminal markers such as MMP-13 and VEGF (Tamamura et al., 2005, Reis and Liebner 2013), while blocking β-catenin signaling leads to a failure of maturation and endochondral ossification in mature chondrocytes and increased chondrocyte death (Mak et al., 2006). Overexpression of β-catenin in cells along the osteochondral junction may stimulate accelerated maturation

and prevent chondrocyte death, while in less mature chondrocytes along the cartilage canals, it may lead to failure of appropriate maturation and endochondral ossification.

Wnt 5b gene expression was significantly increased in chondrocytes around cartilage canals while Wnt 11 gene expression was significantly decreased in foals with OC. Wnt 5b has been shown to be expressed following Ihh expression and may mediate the effects of Ihh (Church et al., 2002). Increases in Wnt 5b expression have been shown to inhibit chondrocyte maturation and terminial differentiation (Church et al., 2002; Yates et al., 2005) and have an opposing role when compared to β- catenin. This opposing role is due to its signal transduction via the non-canonical Ca2+-calmodulin-PKC pathway which can inhibit β- catenin signaling and thus delay terminal chondrocyte differentiation. Wnt 11 expression was significantly decreased in chondrocytes surrounding cartilage canals and has been shown to also inhibit canonical signaling.Wnt11 may mediate the effects of Ihh signaling similarly to Wnt 5b and underexpression would allow for normal maturation of chondrocytes (Church et al., 2002)

Gene expression of LRP6 was significantly increased in chondrocytes surrounding cartilage canals in foals with OC. Binding of specific Wnt proteins, such as Wnt4, to LRP6 complexed with Fzd receptors leads to canonical signaling and expression of LEF/TCF genes. Blocking of LRP6 leads to decreased bone formation, while increased expression leads to increased bone mass (Milat and Ng 2009). Over expression of LRP6 alone has been shown to be able to activate the canonical signaling pathway irrespective of a Wnt signal being present. Increased gene expression of LRP6 correlates well with the increased β-catenin gene expression even in the absence of a significant

increase in Wnt 4 gene expression. These findings indicate increased canonical signaling in chondrocytes along cartilage canals in OC foals.

Increased gene expression for multiple inhibitors of canonical Wnt signaling (Axin1, Wif1, and Dkk1) was observed in chondrocytes along cartilage canals. Wif 1 binds to Wnt proteins directly to inhibit interactions between Wnt proteins and their transmembrane receptors extracellularly (Milat and Ng 2009). Wif 1 has been identified as a marker of osteoblast maturation (Milat and Ng 2009) but its role in endochondral ossification has yet to be elucidated. It can be presumed that since Wif 1 inhibits canonical signaling, increased expression would decrease β- catenin levels and inhibit chondrocyte maturation. It is not known if increased Wif 1 gene expression is a primary alteration or response to increased β- catenin levels in OC cartilage.

In contrast to Wif 1, Axin 1 is a scaffolding protein that modulates Wnt/β- catenin signaling by binding to β-catenin intracellularly. β-catenin, upon being dephosphorylated following Wnt binding to LRP6 (Dao et al., 2010), dissociates from Axin1 and is able to signal through LEF/TCF transcription factors. In this study, Axin1 gene expression was significantly higher in chondrocytes along the cartilage canals in OC foals. Increased expression of Axin 1 could lead to further suppression of β- catenin and has the potential to delay maturation and hypertrophy of chondrocytes.

Dkk1 is the most studied of the Wnt inhibitory proteins and has a distinct mechanism through which it inhibits Wnt signaling. Dkk1 binds to LRP5/6 causing endocytosis of LRP5/6, thus, removing the Wnt receptor from the plasma membrane (Kawano and Kypta 2003) and decreasing canonical signaling. Mice engineered to lack Dkk1 had increased bone formation and bone mass (Morvan et al., 2006). In contrast,

high Dkk1 levels inhibited Wnt-induced alkaline phosphatase expression (Milat and Ng 2009), suggesting that Dkk1 inhibits chondrocyte maturation and hypertrophy. Misexpression of Dkk1 in endothelial cells was shown to cause defects in endochondral ossification caused by the inhibition of angiogenesis in developing bone and subsequent inhibition of apoptosis of hypertrophic chondrocytes and cartilage resorption (Oh et al., 2012). In our study, mean Dkk1 gene expression in cartilage canal chondrocytes in OC foals was 20 times the mean gene expression in normal controls. This overexpression has the potential to significantly inhibit β-catenin-induced chondrocyte maturation and bone formation (Diarra et al., 2007), suggesting that Dkk1 may play a major role in osteochondrosis of foals. Although our gene expression data suggests an involvement of Dkk1, our protein expression data did not show significant differences in protein expression in chondrocytes around cartilage canals between OC foals and normal controls. Interestingly, overexpression of PTH can activate the Wnt pathway despite overexpression of Dkk1 (Guo et al., 2010). PTH-rP binds to the same receptor as PTH and has been shown previously to have increased gene expression in chondrocytes surrounding cartilage canals (Riddick et al 2012). The elevated Dkk1 gene expression may be in response to increased Wnt signaling due to PTH-rP activation.

The Wnt/β- catenin and retinoic acid receptor signaling pathways interact to regulate chondrocyte function and matrix turnover (Yasuhara et al., 2010). Activation of both is known to tilt cartilage matrix homeostasis toward catabolism. Increased retinoic acid in chondrocytes increases Wnt/β- catenin signaling (Yasuhara et al., 2010). In contrast to our finding of increased SC-PEP gene expression decreased expression of SC-PEP and retinoic acid receptor γ was seen in yearling thoroughbreds with OC as

determined via blood gene expression equine microarray profiles (Bertone and Reed, 2008). This difference may reflect the advanced nature of the disease in yearlings compared to the early lesions in our study, the different methods used to determine gene expression, or it may be a reflection of the differences in gene expression of leukocytes vs. chondrocytes. Although the retinoic acid pathway has been shown to regulate skeletogenesis and chondrogenesis, the role of SC-PEP has not yet been elucidated. SC-PEP has been used to screen for genes induced by retinoic acid (Kollmann et al., 2009). Its increased gene expression suggests increased retinoic acid pathway activation even though our data did not show a significant increase in retinoic acid receptor γ. Increased signaling through the retinoic acid pathway may be an initiating factor in foal OC and down regulation may occur as the disease process progresses.

Although our data showed many significant differences in gene expression of components of the Wnt signaling pathways, there were no significant changes in any of the three secreted frizzled related proteins (Sfrp1, 3 and 5). Sfrp1 has been shown to regulate the appropriate biological timing of development of the hypertrophic chondrocyte phenotype (Gaur et al., 2006). In Sfrp1 knockout mice, there is an acceleration of endochondral ossification and accelerated differentiation of hypertrophic chondrocytes. If timing of Sfrp1 expression is incorrect, then this could lead to abnormal chondrocyte differentiation (Gaur et al., 2006). However, because no significant differences were found in the gene expression of Sfrps in OC foals of our study, these proteins are less likely to be involved in disease pathogenesis.

Cartilage canals have been shown to be involved in the etiopathogenesis of osteochondrosis (Carlson et al., 1995; Shingleton et al., 1997; Olstad et al., 2008).

Looking at our data and previous data (Riddick et al., 2013), the number of significant gene expression differences between chondrocytes associated with cartilage canals in foals with OC and normal foals provides further evidence of the involvement of cartilage canals in the etiopathogenesis of osteochondrosis. Cartilage canals of foals with early osteochondrosis were shown to contain viable blood vessels retained below the ossification front surrounded by small, non-hypertrophic chondrocytes suggesting that patent blood vessels within the cartilage canals may allow for continued exposure of these chondrocytes to signaling molecules preventing appropriate chondrocyte maturation (Shingleton et al., 1997). Also, the patency of these vessels may prevent chondrocyte exposure to hypoxia. Hypoxia induces signaling via hypoxia-inducible factors and may play an important role in chondrocyte hypertrophy, maturation, and death. Hypoxia factors combine with β-catenin to promote chondrocyte apoptosis and endochondral ossification (Wu et al., 2012). In the absence of hypoxia, β-catenin signaling may not be able to induce appropriate chondrocyte death leading to retained cartilage cores along the osteochondral junction.

Although this study provides evidence of aberrant Wnt signaling in chondrocytes surrounding cartilage canals and may be a component involved in the etiopathogenesis of OC, previous studies have suggested that trauma to or focal failure of cartilage canals to provide adequate blood supply to the articular-epiphyseal cartilage complex during development may lead to ischemic necrosis of growth cartilage. In theory, this ischemic growth cartilage would not undergo normal bony conversion, represent a focal disturbance in endochondral ossification, and lead to OC (Carlson et al., 1995; Olstad et al., 2008). Cartilage canals traverse the osteochondral junction which may predispose

these small vessels to injury. The osteochondral junction is where forming subchondral bone attaches to the growth cartilage. This transition zone could represent a significant stress-riser in the growing epiphyseal complex predisposing this area to micro-trauma. Cartilage canals do not form anastomoses with one another and therefore collateral circulation in growth cartilage does not exist (Ytrehus et al., 2007). Damage to one cartilage canal at this stress-riser region could in theory lead to focal ischemic necrosis and OC. The question becomes why some foals have a predisposition for OC and others do not? Altered molecular signaling could lead to a weakened articular-epiphyseal complex, increasing the stress-riser and predisposing their cartilage canals to traumatic injury and OC.

Despite significant differences in gene expression of chondrocytes associated with cartilage canals and the osteochondral junction, statistical analysis of total cartilage, cartilage canal, and osteochondral junction protein expression scores of OC and normal foals did not show any significant differences. Wnt 11, Wnt4, and Dkk1 are small, secreted proteins that exist mostly extracellularly and immunohistochemistry techniques in this study were focused on grading positive chondrocytes and not the extracellular matrix. This may have led to insufficient sensitivity or specificity and prevented detection of statistically significant differences in protein expression. Also, the antibodies used were specific for human analogues and, although the Wnt family of proteins is highly conserved between species and Western blots were performed to validate these antibodies for equine protein, non-specific binding may have occurred. Similar to what laser capture microdissection has done to sensitivity and specificity in the detection of gene expression differences, in the future more sensitive and specific protein analytical

methods such as matrix-assisted laser desorption-ionization time of flight mass (MALDI-TOF) spectrometry and the development of anti-equine Wnt antibodies may be helpful in determining the correlation between gene expression and protein expression of Wnt signaling in foals with OC compared to normal controls.

Future studies should be directed at measuring gene expression levels of other Wnt signals and components of the Wnt signaling pathway. Also, Wnt signals appear to have different effects depending on the maturity level of a given chondrocyte population. In order to better understand the results of the current study, it will be important to determine the relative gene expression levels of collagen X and alkaline phosphatase of the laser captured samples. Determination of what stage these chondrocytes are in may help elucidate the role Wnt signaling plays in endochondral ossification.

Chapter 5

Conclusion

The Wnt signaling network is a very complex pathway involved in development and the maintenance of homeostasis and has been demonstrated in the pathogenesis of a variety of diseases. The pathway has very complex interactions with a number of other important developmental pathways and has been shown to have a key role in the process of endochondral ossification (Milat and Ng 2009). In this study, the protein and gene expression of many of the key members of the Wnt signaling network involved in the complicated process of endochondral ossification were evaluated for their involvement in OC of foals. Although other predisposing factors have been implicated in the development of OC such as increased nutritional plane, rapid growth rate, genetics, and trauma, this study and others have shown alterations in multiple molecular factors and their possible involvement in OC (Mirams et al. 2009; Semevolos et al, 2001;2002;2005; Nixon et al 2008; Riddick et al., 2013). This study also stresses the importance of studying foals prior to the onset of clinical signs of OC and of using LCM technology. Early changes in factors at the osteochondral junction and cartilage canals prior to the onset of clinical OC is more likely to indicate causality as opposed to a secondary change.

We hypothesized that gene and protein expression of the components of the canonical Wnt pathway in chondrocytes of the osteochondral junction and cartilage canals would be increased in early OC lesions when compared to normal controls. Based on our data, we found significantly increased gene expression of β-catenin, Wnt 5b, Wif1, Lrp6, Axin 1, Dkk1, and SC-PEP and significantly decreased Wnt 11 gene

expression in laser captured chondrocytes next to cartilage canals. In laser captured chondrocytes along the osteochondral junction gene expression of β- catenin and SC-PEP was significantly increased in OC foals when compared to normal controls. Protein expression of Wnt 11 in the superficial cartilage layer and Dkk1 protein expression in the deep cartilage layer was significantly decreased in foals with early OC.

In conclusion, our findings supported our hypothesis that Wnt and retinoic acid pathways are altered in early OC and may be associated with disease pathogenesis. Wnt signaling has been shown to be downstream of Ihh signaling and modulates Ihh signaling during endochondral ossification. Since Ihh signaling was previously shown to be altered in laser captured chondrocytes associated with cartilage canals in OC, it makes sense that Wnt signaling should be altered, which was supported by our data. Although interactions between Ihh, BMP, Wnt, and retinoic acid pathways have been demonstrated, the exact timing, factors, and relative expression necessary for appropriate endochondral ossification has not been elucidated. What we do know is that all of these pathways have a role and the complex interactions are carefully coordinated allowing for endochondral ossification. The Ihh gene expression (Riddick et al., 2013) and Wnt gene expression differences between OC foals and normal controls in chondrocytes specifically associated with cartilage canals provides further evidence that cartilage canals are involved in the etiopathogensis of osteochondrosis. Future studies directed to improve our knowledge of the complex interactions of signaling pathways involved in endochondral ossification and further evaluation of other signaling molecules associated with Wnt signaling will be important to improve our understanding of the etiopathogenesis of OC.

Footnotes

a. Tissue Tek OCT compound, VWR International, West Chester, PA
b. Oregon Health Sciences University
c. Cryojane, Instrumedics, Hackensack, NJ
d. Arcturus Bioscience, Mountain View, CA
e. Applied Biosystems, Foster City, CA
f. Agilent Technologis, Palo Alto, CA
g. Qiagen, Valencia, CA
h. StepOne plus, Applied Biosystems, Foster City, CA
i. Biogenex, San Ramon, CA
j. Spex Certi Prep, Metuchen, NJ
k. Trizol, ® Invitrogen, Carlsbad, CA
l. NanoDrop ND 1000, Thermo Fischer Scientific, Wilmington, DE
m. Invitrogen, Carlsbad, CA
n. Thermoscientific, Rockford, IL
o. Sigma-Aldrich, St. Louis, MO
p. Santa Cruz Biotechnology, Inc., Dallas, TX
q. R&D systems, Minneapolis, MN

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