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Early Detection and Intervention Strategies for Acute Cartilage Injury after Intact Joint Impact: In-vitro Porcine Knee Model

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EARLY DETECTION AND INTERVENTION STRATEGIES FOR ACUTE CARTILAGE INJURY AFTER INTACT JOINT IMPACT: IN-VITRO PORCINE KNEE MODEL

By

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Traumatic injury to cartilage has been shown to lead to Post-traumatic Osteoarthritis (PTOA). The acute phase of PTOA is characterized with increased expression of aggrecanases and inflammatory cytokines in the injured cartilage. Aberrant regulation of microRNAs (miRNAs) in the cartilage has been associated with the pathogenesis of PTOA. The objective of this dissertation work was to elucidate potential early detection and intervention strategies for acute knee injury based on the miRNA and genetic changes of knee joint tissues. An ex-vivo intact joint impact injury model was created to examine the miRNA and inflammatory and degenerative gene expression in cartilage and meniscus during the acute phase of injury. Using this model, four potential early intervention treatments for PTOA were compared, and their effects on the early inflammatory and catabolic events after acute cartilage injury were determined at 8 hours after intact joint impact. The time- and concentration-dependent nature of cellular and extracellular miRNAs in chondrocytes, synoviocytes, and meniscus cells as influenced by inflammatory cytokines and the ratio of extracellular miRNA were analyzed as potential indicator of early OA progression. In a proof of concept study, the ratios of synovial fluid miRNAs after acute cartilage injury were examined using the ex-vivo intact joint impact model as potential indicator of acute cartilage injury. A recommendation for further study is enclosed.

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Chapter 1 Introduction

1.1 Post-Traumatic Osteoarthritis

With today's ever-increasing active lifestyle, injuries to the knee and other articulating joints of the body are also increasing. These injuries, either through sports, accidents, or military combat, can include a ligament tear, a meniscal tear, damage to the articular cartilage, or any combination of the three in the knee specifically. A study conducted over 14 years following soccer player injuries [165] reported that 12 to 14 years after an anterior cruciate ligament (ACL) tear, approximately 50% of the individuals showed radiological signs of osteoarthritis (OA). This phenomenon has been termed Post-Traumatic Osteoarthritis or PTOA and is also seen in individuals who previously suffered meniscal injury [16] and hard impact to the cartilage [131].

In general, OA refers to the degeneration of cartilage at the articulating surfaces of bones to the point, in the case of severe OA, that the bones are in direct contact and grind against each other. This debilitating and painful orthopedic disease is the most common joint disease, affecting over 20 million people in the United States [54], and places a huge socioeconomic burden on the healthcare system [18]. Popular belief portrays OA as a disease that only affects the elderly or middle-aged people. But because cartilage degeneration can occur within 15 years of a traumatic knee injury, PTOA has become more prevalent in young individuals (ages 35-54) and is responsible for up to 12% of OA cases[22]. The annual socioeconomic liability of PTOA alone in the US is estimated to be $3.06 billion[22].

1.2 Articular Cartilage and Osteoarthritis

The biology of the knee joint tissues varies greatly in cell type, structure, and function, yet they all work cohesively to effectively withstand static and dynamic loading on the knee. Articular cartilage and meniscus are predominantly avascular in nature and serve to dissipate repetitive compressive loading. Located at the ends of bones in joints, such as the knees, articular cartilage enables the bones of a joint to easily glide over one another with very little friction. It also acts as a cushion between joints by distributing the load of pressure and weight over the surface of the joint. Cartilage contact stresses generally range from 2 to 12 MPa, or possibly higher[2].

The ability of cartilage to withstand such high magnitudes of loading can be attributed to its structure. Cartilage can be considered as composed of two immiscible phases: a solid phase (~20%) and a fluid phase (~80%)[92]. The solid phase is represented by a type II collagen (50-73% dry weight) and proteoglycan (15-30% dry weight) matrix. Chondrocytes only comprise 3-5% of total cartilage mass. The fluid phase is represented by water (58-78% wet weight) and dissolved inorganic salts that saturate the solid matrix [142]. Each phase in the cartilage tissue contributes to its intrinsic material properties.

The solid phase of cartilage comprises of a mostly type II collagen and proteoglycan matrix. The most important mechanical properties of the collagen fibers are their tensile stiffness and strength [174]. The density of the fibers, the fiber diameter and orientation, and the amount of cross-links contribute to the overall mechanical stability and high tensile strength of the collagen network [141]. Proteoglycans constitute the second largest portion of the solid phase. In cartilage, they consist mainly of the large aggregating type and large non-aggregating proteoglycans, also known as aggrecans, with

distinct small proteoglycans also being present[134]. Most importantly, aggrecans contribute significantly to the compressive and swelling properties of cartilage by virtue of their abundant charge and hydroxyl groups able to entrap up to 50 times their weight in water[150].

In normal uninjured cartilage, chondrocytes maintain homeostasis by synthesizing these necessary matrix proteins while also producing the matrix-remodeling enzymes aggrecanase and collagenase. Upon the onset of OA by impact injury or age, chondrocytes begin to synthesize more matrix-degrading enzymes than matrix-building proteins[91]. This imbalance results in a compromised cartilage structure with reduced mechanical stability. Furthermore, because cartilage has little to no vascularization, the tissue has limited capacity for healing via cartilage-progenitor cells or nutrition. This eventually leads to further degeneration of the cartilage, loss of matrix and chondrocytes, and fully developed OA.

1.3 Knee Meniscus and Osteoarthritis

The knee meniscus is similar to cartilage in its load bearing function as well as its composition: 72% water and 28% ECM and cells[128]. Again, like cartilage, this highly hydrated tissue is a result of the collagen and aggrecan structural proteins that entrap water molecules to create the necessary fluid pressure that counters the compressive, tensile, and shear stresses acting on the meniscus. In combination, these indispensable matrix proteins and the unique-wedge shape give the meniscus a compression and shear modulus of approximately 100-250kPa each and a tensile modulus of 100-300MPa[154,39].

Unlike cartilage however, meniscus serves a crucial role in shock absorption, load transmission, lubrication, and nutrition for the articular cartilage[23]. The meniscus

achieves these roles through region-specific innervation, vascularization, and cell phenotypes. Throughout development, vascularization in the meniscus decreases substantially, starting fully vascularized at birth to just 10-25% in the periphery of a mature meniscus[29]. The discrepancy in blood circulation in the inner regions of the meniscus correlates directly with its limited healing capacity, leaving it more vulnerable to irreparable post-traumatic degeneration or lesions than the vascularized peripheral regions[7]. The phenotype of meniscus cells also changes from the peripheral region to the inner region, resulting in slight differences in matrix structure. The outer peripheral zone is comprised of fibroblast-like cells which are suspended in an ECM made up mostly collagen type I, similar to the properties of fibrocartilage[97]. In contrast, the inner zone is comprised mostly of round chondrocyte-like cells in a collagen type II matrix, similar to the properties of articular cartilage[101]. Therefore, it is in the inner zone of the meniscus with little to no vascularization, a mostly collagen type II matrix, and chondrocyte-like cell phenotype that is believed to behave similarly, but not identically, to cartilage after a traumatic injury.

Meniscal tears account for the largest proportion of intra-articular knee injuries in the United States[108], and young patients due to sports- or vehicle-related injuries make up one-third of all reported cases[11]. Injury to the meniscus either by impact or a tear causes an imbalance of catabolic enzymes, much like what is seen in cartilage, onset by the excessive mechanical loading and pro-inflammatory environment[67]. The meniscal matrix begins to degrade due to the overproduction of catabolic enzymes and lacks the ability to perform its crucial roles of load distribution and joint lubrication. Inevitably, the altered mechanical loading of the entire knee joint elevates the risk of OA development[34].

1.4 Synovial Membrane, Synovial Fluid, and Osteoarthritis

The synovial membrane encapsulates the knee joint with attachments at the femur and tibia and is the main source of synovial fluid which allows for frictionless movement across articulating surfaces[59]. Unlike the other two tissues, the highly vascularized nature of the synovial membrane makes it the primary site for nutrient exchange. This one to three cell layer thick membrane allows oxygen and nutrients, such as glucose, to enter the joint capsule and nourish the other knee joint tissues via the synovial fluid[59]. The synovial fluid allows for paracrine interactions between tissues in the enclosed joint capsule. After a traumatic joint injury, cell fragments and ECM degradation products are released into the surrounding synovial fluid[153]. The breakdown products cause the membrane to become inflamed, also known as synovitis, and can incite the synovial membrane to produce matrix-degrading enzymes[112]. Induction of the inflammatory cytokines synovial interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF- α) is also up-regulated in synovitis and early OA which accelerates the progression of the disease[130]. The enclosed nature of the joint microenvironment which encapsulates three major and diverse tissues allows for unique interactions amongst them. The condition of an injured and inflamed joint then becomes multi-faceted and complex with more than one tissue contributing to the development of PTOA.

1.5 Osteoarthritis Pathogenesis

While the pathophysiology of PTOA still remains poorly understood, the disease displays the classic OA symptom of cartilage degeneration, led by an over-production of catabolic enzymes. The most prominent enzymes in cartilage matrix degradation are collagenase-3 (matrix metalloproteinase 13 [MMP-13]), stromelysin-1 (MMP-3),

aggrecanase-1 (A Disintegrin And Metalloproteinase with Thrombospondin motifs 4 [ADAMTS-4]), and aggrecanase-2 (ADAMTS-5). MMP-13 cleaves a broad range of substrates including collagen, aggrecan, and fibronectin, but its highest specificity, and therefore potency, is to collagen type II[69]. Additionally, MMP-3, which cleaves proteoglycans and is highly expressed in OA, activates pro-MMP-13, furthering matrix breakdown[30,78]. ADAMTS-4 and ADAMTS-5 have the highest substrate specificity to the proteoglycan aggrecan, each cleaving the protein at its specific respective site along the core[88]. After injury[8,9] or in the presence of inflammatory cytokines IL-1β and TNF-α[160,4,96,30,86], the genetic expressions of the MMP-13, MMP-3, ADAMTS-4, and ADAMTS-5 are up-regulated in the injured tissues or cells, indicating an activation of matrix-degrading enzymes by the injured cells themselves. In contrast, expressions of collagen type II and aggrecan production are down-regulated, indicating an imbalance of catabolic and anabolic activities[8]. Chondrocyte necrosis after injury contributes to the decrease in matrix synthesis[9]. Again, due to its intrinsic poor nutrient supply, the limited regenerative capacity of cartilage exacerbates the degeneration. With the structural integrity of the articular cartilage compromised due to degeneration, the tissue is unable to adequately perform its function of resisting compressive loads during joint articulation.

1.6 MicroRNA Regulation and Osteoarthritis (Table 1)

Genetic regulation of the catabolic enzymes and anabolic proteins that characterize OA begins in the cytosol of the chondrocyte by ~20-nucleotide non-coding RNAs called microRNAs (miRNAs)[77]. In their simplest form, miRNAs negatively regulate their target messenger RNA (mRNA) post-transcriptionally by promoting degradation, deadenylation, or repression of translation [12]. What makes these small

nucleic acids so remarkable is that a single miRNA can target multiple genes involved in the same or different biological process and induce modest epigenetic changes and altered transcription factor activity. Dysregulation of miRNA expression, however, has been shown to cause disease by interfering with the regular homeostasis expression of their target genes[37] (Figure 1.1). Specifically, the importance of miRNA in cartilage homeostasis and maintenance was exemplified in mice lacking the endoribonuclease *Dicer*, which converts non-functional pre-miRNA into mature, functional miRNA[48,70]. This study witnessed reduced limb size and body mass in *Dicer*-knockout mice as compared to their normal control as a result of unregulated chondrocyte growth and eventual chondrocyte necrosis[70].

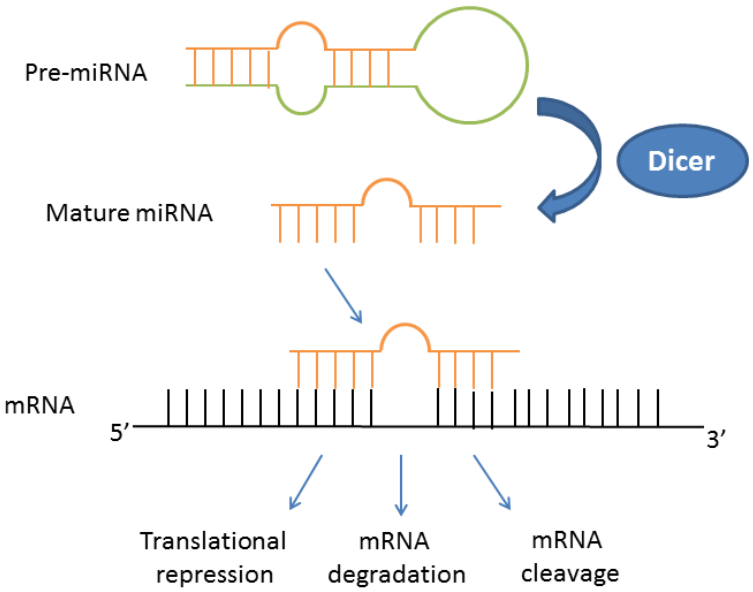

Figure 1.0.1 - miRNA mechanisms for gene regulation. Some miRNAs bind to mRNA targets with perfect complementarity to enduce degradation or mRNA cleavage. Other miRNAs can bind to targets with imperfect complementarity and repress translation.

Other recent studies have demonstrated the importance of several miRNAs in cartilage homeostasis and the progression of PTOA. Expression of collagen II (COL2A1) is known to be controlled indirectly by several miRNAs, such as miR-148a, miR-145,

miR-675, and miR-101. In human OA chondrocytes via viral transfection, overexpression of miR-148a increased expression of COL2A1 and decreased expression of fibrocartilage, COL1A1[166]. MiR-145, miR-675, and miR-101 regulate the production of COL2A1 through the major chondrocyte regulation transcription factor SOX9[32,33,95]. SOX9 has been identified as critical to the chondrocyte-specific expression and production of COL2A1 and, therefore, cartilage formation[79]. However, the expression of SOX9 is down-regulated in human osteoarthritic cartilage, contributing to the weakened structural matrix characteristically seen in OA[45]. Up-regulated in OA cartilage and in IL-1β-treated chondrocytes, miR-145 and miR-101directly inhibit production of SOX9, and their over-expression resulted in greatly reduced expression of matrix proteins COL2A1 and aggrecan[32,95]. MiR-675, down-regulated in IL-1β-treated chondrocytes, has been proposed to indirectly regulate COL2A1 expression by targeting a COL2A1 repressor protein[33,146]. In this de-repression mechanism, SOX9 up-regulates miR-675, which then down-regulates the COL2A1 repressor, and, therefore, up-regulating COL2A1 expression. Additionally, transforming growth factor β (TGF-β) is recognized as a critical component to the proper differentiation and maintenance of articular chondrocytes[163]. MiR-455 has been implicated in the regulation of TGF-β signaling via the Smad2/3 pathway[155]. Up-regulation of miR-455, seen in human OA cartilage, results in a decrease in TGF-β signaling which can then lead to cartilage loss.

With regards to catabolic processes, there are seven different miRNA that have been shown to regulate MMP-13, either directly or indirectly. MiR-27b and miR-127 directly regulate MMP-13, expressing decreased regulation of miRNAs with increased production of the collagenase enzyme in OA cartilage[4,123]. MiR-9 also directly regulates

MMP-13 expression but is shown to be significantly up-regulated in OA chondrocytes[64]. It is theorized that miR-9 may act in a chondroprotective mechanism. MiR-22, miR-140, miR-488, and miR-558 indirectly regulate MMP-13 activity by targeting various enzyme-influencing proteins. MiR-22 regulates PPARA and bone morphogenic protein-7 (BMP-7), two key receptor and signaling proteins for cartilage inflammation and homeostasis[57]. Up-regulation of miR-22, as seen in OA cartilage and IL-1β-treated chondrocytes, corresponds with up-regulation of IL-1β and MMP-13 and down-regulation of aggrecan synthesis[57]. Additionally, when treated with miR-22 inhibitors, osteoarthritic chondrocytes had increased expression of PPARA and BMP-7, decreased IL-1β and MMP-13 activity, and increased aggrecan production. A prominent factor in cartilage homeostasis[106], miR-140 acts as a negative-feedback regulator of MMP-13, indirectly modulating the inflammatory NF-κB pathway and regulating the enzyme in OA[86]. Up-regulation of miR-140 in OA and IL-1β-treated samples[86,155] has been associated with MMP-13 production. Significantly decreased in OA chondrocytes, miR-488 and miR-558 indirectly regulated MMP-13 by targeting the ZIP-8 zinc transporter [144] and inhibiting the activation of the NF-κB pathway[122], respectively. Zinc Zn^{2+} is critical to the catalytic activity of MMP-13[120], and down-regulation of miR-488 functions to create a local environment of increased Zn^{2+} concentration, propagating MMP-13 activation[144]. Overexpression of miR-558, meanwhile, suppressed IL-1β-mediated MMP-13 expression by suppressing activation of the NF-κB pathway[122].

Table 1 Potential miRNAs for Cartilage Regeneration, Inflammation Reduction, Pain Reduction, and Apoptosis Inhibition.

miRNAs for Cartilage Regeneration	Target gene/Function	Expression in OA Cartilage/ IL-1β-treated chondrocytes
miR-148a	Overexpression increases COL2A1, decreases COL1A1[166]	Decreased [166]
miR-140	ADAMTS-5[106], MMP-13[86]	Decreased [106], Increased [86,155]
miR-125b	ADAMTS-4[96]	Decreased [96]
miR-127	Up-regulates MMP-13 by IL1b[123]	Decreased [123]
miR-22	Regulates PPARA and BMP7[57]	Increased [57]
miR-9	Targets protogenin (PRTG) [143]; Targets MMP-13[64]	Decreased [143]; Increased [64]
miR-27b	MMP-13[4]	Decreased [4]
miR-488	Directly regulates zinc transporter, indirectly regulates MMP-13; degeneration by IL-1β stopped by miR-488[144]	Decreased [144]
miR-558	Overexpression inhibits IL-1β induced MMP-13 and NF-Kβ [122]	Decreased [4,122]
miR-675	SOX9 positively regulates COL2A1 via miR-675[33]; H19/mir675 positively regulates COL2A1[146]	Decreased [33,146]
miR-455	SMAD2, ACVR2B, CHRDL1, TGF-β signaling [155]	Increased [155]
miR-145	SOX9[95]	Unknown
miR-101	Regulates SOX9; prevents ECM synthesis[32]	Increased [32]
miR-17	Targets TIMP1/2[82]	Increased in disease (observed in mouse cardiomyocytes)[82]
miRNAs for Inflammation Reduction	**Target gene/Function**	**Expression in OA Cartilage/ IL-1β-treated chondrocytes**
miR-146a	Negatively regulates IRAK1, TRAF6, and NF-κB signaling[156,168]	Increased [81,177]
miR-22	Regulates PPARA and BMP-7[57]	Increased[57]
miR-149	May regulate TNF-α[136]	Decreased[136]
miRNAs for Joint Pain Reduction	**Target gene/Function**	**Expression in OA Cartilage/ IL-1β-treated chondrocytes**
miR-146a	Modulates TNFα, COX-2, iNOS, IL-6, IL8, RANTS and ion channel,	Increased[83]

	TRPV1[83]	
miR-558	Regulates COX-2[122]	Decreased[122]
miR-199a	Regulates COX-2 [3]	Decreased[3]
miRNAs for Chondrocyte Apoptosis Inhibition	**Target gene/Function**	**Expression in OA Cartilage/ IL-1β-treated chondrocytes**
miR-34a	Regulates XIAP [117]	Increased [1,117]
miR-15/miR-16	Regulates BCL2 [27]	Increased (in B cell lymphoma) [27]
miR-9	Targets PRTG; Inhibition increases apoptosis and CASP3 activity [143]; Targets MMP-13[64]	Decreased[143]; Increased [64]

Tissue Inhibitor of Metalloproteinases (TIMP) inhibits the function of MMPs at the enzymatic level in order to maintain the proper regulation of catabolic processes[38]. Expression of TIMP-1, direct inhibitor of MMP-13, has been shown to be significantly down-regulated in OA cartilage – another factor influencing the increase in collagenase activity[66]. In a study on mouse cardiomyocyte remodeling, miR-17 was shown to directly regulate TIMP-1 and 2 expressions[82]. Supplementation of miR-17 effectively increased production of TIMP 1 and 2 and thereby decreased MMP production. MiR-125b and miR-140 directly target ADAMTS-4 and ADAMTS-5, respectively[106,160]. Both miRNAs are significantly down-regulated in OA cartilage and IL-1β-treated chondrocytes, resulting in loss of extracellular proteoglycans, a characteristic indicator of early OA progression.

OA is widely accepted as an inflammatory disease[15,43,60] with contributing symptoms such as matrix degradation, pain, and cellular apoptosis as a result of that inflammation. In an osteoarthritic knee, inflammatory cytokine concentration increases in the synovial fluid as well as in the diseased cartilage[17]. IL-1β and TNF-α [17,53] trigger a catabolic pathway, disrupting cellular homeostasis. Various miRNA have been

implicated with regulation of OA inflammation. MiR-146a is highly influential in the cellular response to inflammatory cytokines through the regulation of both the IL-1 receptor-associated kinase 1 (IRAK1) and the TNF receptor-associated factor 6 (TRAF6) genes[156]. IRAK1 and TRAF6 are key proteins in the IL-1 receptor signaling cascades, mediating activation of the NF-κB pathway[62,179]. In patients exhibiting low grade OA symptoms, as well as in *in-vitro* IL-1β-treated chondrocytes, miR-146a was shown to be significantly up-regulated[81,156,168,177]. MiR-22 negatively regulates the proteins BMP-7 and PPARA which regulate IL-1β and MMP-13 expressions, and its expression is significantly up-regulated in OA cartilage[57]. While both miRNA are up-regulated in OA, up-regulation of miR-146a attempts to inhibit the inflammatory signaling cascades, while up-regulation of miR-22 promotes inflammatory pathways. MiR-149 has been shown to have a direct regulatory effect on TNF-α production, and its expression is significantly decreased in OA chondrocytes[136].

Joint pain due to symptomatic OA is often the first indication of disease to a patient. When left untreated, the pain caused by OA significantly lessens quality of life and reduces the ability to perform the basic activities of daily life[145]. Osteoarthritic joint pain has been associated with the production of Cyclooxygenase (COX)-2 and Prostaglandin E_2 (PGE_2) proteins[84]. In healthy non-diseased chondrocytes, COX-2 and PGE_2 are not constitutively expressed in detectable amounts, but, after induction with inflammatory cytokines or in OA cartilage, expressions of both proteins are significantly up-regulated – up to 50-fold higher than levels in normal cartilage[125]. Researchers have discovered that miR-199a and miR-558 directly negatively regulate COX-2 in chondrocytes and their expressions are significantly decreased in OA cartilage[3,122].

However, only overexpression of miR-558 significantly inhibited IL-1β-induced COX-2 protein expression[122]. Overexpression of miR-146a in IL-1-treated chondrocytes, synoviocytes, and glial cells suppressed catabolic enzyme activity, regulated inflammatory factors, and reduced COX-2 and other pain-related molecules[83].

The cartilage matrix relies solely upon the chondrocytes within the tissue for maintenance and remodeling, even though chondrocytes comprise less than 5% of the tissue mass[1]. Chondrocyte viability is, therefore, critical to the structure and function of healthy cartilage tissue. Studies have shown that impact injury and OA induces apoptosis in chondrocytes[49,50,164] which reduces cell number and hinders the ability of cartilage to repair itself. Inhibition of miR-34a, which is up-regulated in OA cartilage[1,117], increases chondrocyte resistance to apoptosis by regulating X-linked inhibitor of apoptosis protein (XIAP)[117]. B cell lymphoma 2 (BCL2) is another apoptosis inhibitor that is commonly overexpressed in cancer cells[135] but its expression is severely down-regulated in OA, promoting apoptosis[173]. The cluster of miRNAs miR-15 and miR-16 has been shown to directly regulate BCL2 expression[27]. Conversely, miR-9 overexpression increases apoptosis inhibition by targeting protogenin (PRTG) and reduced cartilage degeneration when injected into experimentally-induced OA mouse knee joints[143].

1.7 Potential Detection and Intervention Strategies for PTOA

Current therapy for PTOA is similar to that of primary OA, with optimal management requiring a combination of non-pharmacological and pharmacological modalities[181]. The limitation of these modalities is that they are for management of the disease once it has already occurred and fail to correct the underlying pathology[31], resulting in continued disease progression. Unlike primary OA, however, PTOA has a

known starting point of disease, namely the injury event, and thus uniquely represents an appealing opportunity for the use of targeted therapy that could prevent or delay the onset of PTOA.

Cartilage fractures and ACL and meniscal tears can be identified immediately after the injury through common radiological methodologies, such as Magnetic Resonance Imaging (MRI) and CT scans, or arthroscopy. The physical tear in the tissue visible through these modalities verifies the occurrence of a traumatic injury. This provides appropriate timing information for the administration of early intervention therapies to prevent the development of PTOA years later. However, understanding of suitable targets for these early intervention therapies is severely lacking in current clinical techniques. Thus, there is a substantial need for a method of early intervention that will effectively prevent or delay the degeneration of cartilage after acute traumatic injury.

Several studies have reported efficacy of cell- and molecular-based strategies in the prevention of matrix degeneration. Mesenchymal Stem Cells (MSCs) have been shown to exhibit immunosuppressive and chondroprotective characteristics in the treatment of OA. Intra-articular injection of MSCs at the early time point of 7 days protected against synovitis and cartilage destruction in an *in-vivo* murine OA model[138,158]. Additionally, MSC intra-articular injection in multiple large animal OA models resulted in greater repaired cartilage area and reduced joint inflammation[5,76,98,114,137].

Interleukin-1 Receptor Antagonist Protein (IRAP) is a naturally-occurring anti-inflammatory cytokine in the body which prevents IL-1α/β from binding to its receptor and triggering its catabolic pathway[99]. Previous studies[60,67,99,111] have shown that intra-articular injection of IRAP reduces catabolic activities and joint discomfort in models of

established OA. Recent studies using a mouse articular cartilage fracture model have shown that IRAP, as an early intervention treatment, significantly reduced cartilage degeneration and synovial inflammation[41,68].

Dexamethasone (DEX) is a potent corticosteroid known for its anti-inflammatory properties[21,65]. Intra-articular injections of DEX have been commonly and effectively used clinically for symptomatic relief in OA patients[14]. More recently, several studies have shown that DEX reduces glycosaminoglycan loss in cartilage injury in vitro, enhances progenitor cells to differentiate into chondrogenic cells and synthesize proteoglycans, and prevents aggrecan degradation[85,93,94,151]. Further, DEX decreased joint inflammation and tissue degradation in an in vivo PTOA model in rabbits [55].

Hyaluronan (HA) is the principle glycosaminoglycan found in synovial fluid and is responsible for viscosity and lubrication within the joint[139,140]. Intra-articular HA therapy has been used as an alternative for the treatment of knee OA pain with beneficial anti-inflammatory and chondroprotective effects[116]. HA is theorized to elicit its therapeutic anti-inflammatory chondroprotective effects by stimulating more endogenous production of hyaluronate[10]. Alternatively, administered HA has been suggested to prevent the degradation of endogenous HA by removing catabolic enzymes and cytokines[35].

After such a traumatic injury, such as high impact loading for example, the ligaments, menisci, and cartilage often appear unharmed after radiological observation. Currently, there are no reliable methods of diagnosing OA before radiological evidence of the disease develops, which hinders the effectiveness of therapies and potential preventative measures. Several studies have investigated the expression of molecular by-

products of cartilage degeneration, such as cytokines and matrix fragments, as potential biomarkers of OA[24,104,152]. Other methods currently under investigation of efficacy include quantification of biochemical markers in the synovial fluid, such as nitrotyrosine, tenascin-C, CD-14, OPN, S100A8/A9 proteins, a range of interleukins, and others[25,47,61,63,89,104,113]. While some of the listed biomarkers showed promise in successfully discriminating between degenerated and healthy knees, they all required the cartilage to have some level of progressive degeneration and most importantly, the tissue source of the biomarkers were unknown. Therefore, there is a concurrent substantial need for a minimally-invasive method for the detection of acute cartilage injury.

1.8 Research Aims and Objectives

Traumatic injury to cartilage has been shown to lead to PTOA. The acute phase of PTOA is characterized with increased expression of aggrecanases and inflammatory cytokines in the injured cartilage. Aberrant regulation of miRNAs in the cartilage has been associated with the pathogenesis of PTOA. Early intervention therapies aim to be administered during the acute phase for the prevention of PTOA development. Furthermore, there is a need for a method for the early detection of cartilage injury prior to radiological evidence of PTOA. The dissertation work that follows attempts to elucidate early detection and intervention strategies for acute knee injury based on the miRNA and genetic changes of knee joint tissues. It is the first step in a continuum of translational research that is expected to lead to the development of optimized diagnostic and therapeutic strategies for injured orthopedic tissues.

The following are the hypotheses of this study:

1. Early intervention with cell- and molecular-based therapies can preserve chondrocyte viability and reduce catabolic and inflammatory expressions in acutely injured cartilage.
2. The expression of synovial fluid miRNAs can be used as a method of early detection of acute cartilage injury.

To test these hypotheses, the following specific aims were created.

Specific Aim 1: To investigate the miRNA expression profile in articular cartilage, synovial membrane, and meniscus after acute injury of articular cartilage

A porcine intact joint impact injury model was developed. A custom impact device was designed and built to create a single impact injury to intact porcine knee joint. Two time points (3hrs and 8hrs) was used to characterize the injury response after 10kg impact. A pressure sensitive film was used to determine the contact area and pressure created by the 10 kg impact for further characterization of the intact joint injury model. Cartilage samples were harvested at respective time point post injury for analyses of cell viability, catabolic gene expression, and inflammatory protein production. MiRNA expressions specifically related to cartilage homeostasis, OA, and ECM breakdown were also analyzed in injured cartilage, meniscus, and synovial membrane samples.

Specific Aim 2: To investigate and compare the effect of cell- and molecular-based (MSC, IRAP, HA, and DEX) treatments on the cell viability and catabolic and inflammatory genetic expression of acutely injured articular cartilage.

The same porcine impact injury model described in Specific Aim #1 was used. One hour after impact with 10kg weight, an intra-articular injection of MSCs, IRAP, HA, or DEX was administered to the knee joint. Cartilage and meniscus samples were harvested at 8 hours post injury for analyses of cell viability, catabolic miRNA and gene expression.

Specific Aim 3: To determine the time- and concentration-dependent nature of cellular and extracellular miRNAs in chondrocytes, synoviocytes, and meniscus cells as influenced by inflammatory cytokines and examine the ratio of extracellular miRNA as a potential indicator of early OA progression.

For time-dependent studies, three cell types were stimulated with 10ng/mL IL-1β or 50ng/mL TNF-α for 8, 16, and 24 hours. For concentration-dependent studies, chondrocytes were stimulated with a higher level of IL-1β (20ng/mL) or TNF-α (100ng/mL) for 8 hours. Cellular and extracellular expressions of miR-22, miR-16, miR-146a, miR-27b, and miR-140 were analyzed by RT-PCR. We examined the ratio of extracellular miRNAs as a method to determine differential expression without the need for an internal control.

Specific Aim 4: To examine the ratios of synovial fluid miRNAs after acute cartilage injury using the ex-vivo intact joint impact model.

The same porcine impact injury model described in Specific Aim #1 was used. Eight hours after impact, synovial fluid samples from injured and control joints were

collected for analyses of miR-22, miR-16, miR-146a, miR-27b, miR-125b, miR-34a, and miR-140 by RT-PCR. Similar to Specific Aim #3, we examined the ratios of extracellular miRNAs to determine an indicator of acute cartilage injury.

Contribution

This contribution will establish a foundation for future studies of methods of early detection and intervention of cartilage injury after a traumatic knee injury in the minimally-invasive manner of intra-articular sampling and injections. Also, better fundamental understanding of miRNA regulation of matrix remodeling is presented, offering the basis of further research into suitable miRNA targets to prevent or retard cartilage degeneration.

Chapter 2
MicroRNA and mRNA Expression in Knee Joint Tissues after Acute Intact Joint Impact Injury

2.1 Background

Traumatic injury to the knee has been shown to lead to Post-Traumatic Osteoarthritis (PTOA)[165]. Cartilage degeneration can occur within 15 years of a traumatic knee injury; therefore, PTOA has become more prevalent in young individuals and is responsible for up to 12% of OA cases[22]. In effort to determine the mechanism of PTOA development, researchers have developed various *in-vitro* and *in-vivo* models of impact injury to cartilage. Monolayer chondrocyte injury models[4,86,96] which use inflammatory cytokines to mimic an injury microenvironment have the advantage of cell number but lack the three dimensional matrix and ability to transduce the mechanical forces associated with impact injuries. Cartilage explant models[8,9,160] have a three dimensional extracellular matrix; however, they are usually maintained in non-physiological culture conditions which contain higher amounts of nutrients and oxygen as compared to the avascular, hypoxic environment within the knee joint. Rundell et al. determined that the true cartilage explant response to mechanical loading is altered by equilibration in standard culture medium.[133] While the *in-vivo* impact injury model[56] remains the "gold-standard" for investigating the true pathophysiological response of cartilage after injury, the *ex-vivo* intact joint injury model utilized in this study may provide a physiological acute injury response in all knee tissue types with minimal disruption of native tissue environment in a more cost-effective manner.

After a traumatic knee injury, with or without cartilage fracture, inflammatory cytokine concentration increases in the synovial fluid and injured tissues[17]. The primary cytokines produced in an inflammatory environment are Interleukin (IL)-1β and Tumor Necrosis Factor (TNF)-α[17,53]. Up-regulation of IL-1β after injury triggers a catabolic pathway, disrupting cellular homeostasis by increasing aggrecanase and collagenase production and decreasing collagen and aggrecan production[60]. This imbalance of anabolic and catabolic processes can be traced back to aberrant epigenetic regulation by microRNAs (miRNAs), ~20-nucleotide non-coding RNAs[77]. In their simplest form, miRNAs negatively regulate target messenger RNA (mRNA) post-transcriptionally by promoting degradation, deadenylation, or repression of translation[12]. Seven miRNAs miR-125b, miR-140, miR-16, miR-22, miR-146a, and miR-27b, among others, have been implicated in the progression of OA and their expressions have been shown to be influenced by an inflammatory environment (see Chapter 1 & Table 1). Additionally, MMP-3, which cleaves proteoglycans and is highly expressed in OA cartilage, activates pro-MMP-13, furthering matrix breakdown[30]. Proper regulation of catabolic enzymes and inflammatory pathways is critical to achieve homeostasis in cartilage after injury. Thus, there is a substantial need for a method of early intervention that will effectively prevent or delay the degeneration of cartilage after acute traumatic injury.

In this study, we utilized an *ex-vivo* intact joint injury model to examine the immediate changes in miRNA expression in cartilage, meniscus, and synovial membrane at 3 and 8 hours after injury. We hypothesized that each of the knee joint tissues will express a tissue-specific miRNA profile of inflammatory, degenerative, and apoptotic genetic markers in the early phase of a single impact injury.

2.2 Materials and Methods

Injury Model Development

A custom impact device was developed to create replicable injury *ex-vivo* to intact porcine knee joint. Porcine legs from 35-40kg pigs were acquired from the University of Miami Department of Veterinary Resources Tissue Sharing program (Institutional Animal Care and Use Committee approved source) and were disarticulated at the hip within 2 hours of death. Skin and muscle surrounding both joints were removed to fit the leg properly in extension in a confined chamber of the impact device, while maintaining the synovial membrane and joint capsule intact. The confined chamber prevented knee flexion upon impact. An impact cap comprised of a polycarbonate tube and metal plate was placed securely over the femoral head to ensure flat impact surface. A knee joint from each pig was randomly selected and subjected to an impact force about 4.6kN by dropping a 10kg weight one time from 1 m directly above the knee in extension (Figure 2.1). The impact force was chosen based on average mass withstood per leg (40kg pig/4 legs = 10kg/leg). The chosen impact force did not result in visible cartilage damage or bone fracture. A force transducer (Omega DLC101 Impact Sensor, Omega Engineering Inc.) was placed below the platform on which the leg stood to assure impact force reproducibility. The contralateral joint of the same pig (control) was placed within chamber of the impact device but was not subjected to impact. Immediately after impact, limbs were wrapped in saline-soaked gauze to prevent dehydration and placed in humidified chamber at physiological temperature 37°C and 20% oxygen until time point. At 1 hour post-impact, control and injury joints received 1mL of sterile Phosphate Buffered Saline (PBS) injections to simulate intra-articular injection.

To determine appropriate end time point for genetic changes to take place, cartilage samples from impacted areas on femoral condyles of control and injury knees were harvested at 3 and 8 hours post-impact, flash-frozen, and stored at -80°C for genetic expression analysis.

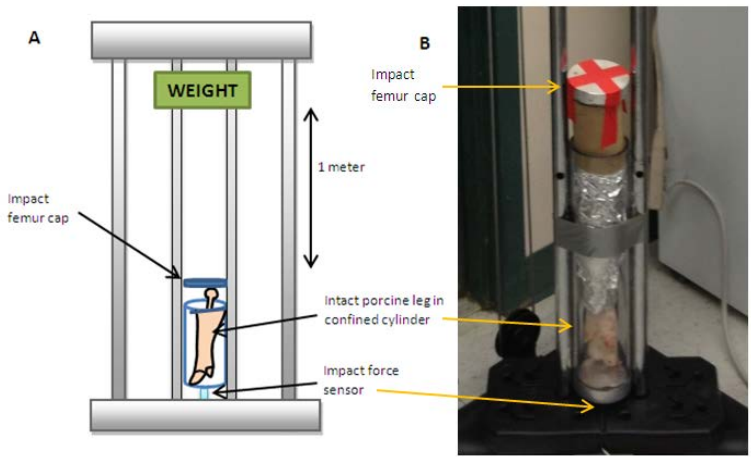

Figure 2.1- Schematic of Intact Joint Impact device

Fuji Film Pressure and Area Analysis

A medium-grade pressure sensitive film (FujiFilm; Sensor Products, Inc. Madison, NJ) was used to determine the contact area and pressure created by the 10 kg impact, as previously described by Clark et al[28]. In brief, the patellofemoral tendon and synovial membrane were dissected to allow access into the knee joint. The film was sealed between thin polyethylene adhesive layers, and 2 strips of 2 x 6 cm^2 were inserted into the femoral-tibial joint in an anterior/posterior direction, separated by the anterior and posterior cruciate ligaments. After impact, digital images of the Fujifilm stain were obtained at a resolution of 600ppi using a digital scanner. To verify the linear relationship between applied pressure and stain intensity, Fujifilm strips underwent a series of four static load tests. Compressive loads, ranging from 15 to 40 MPa, were applied by a

3.36mm diameter cylindrical indentor using an Instron materials-testing machine (Instron Co., Norwood, MA).

Each Fujifilm stain image underwent initial processing to account for the granular texture of the film (MATLAB 2014a)[87]. Images were divided into 4x4 pixel areas. The mean intensity of the pixels in the area was then applied across the entire 4x4 pixel area. Pressures calculated from standard curve were applied to impacted stains to produce a pressure distribution map. Injured area defined by pressures >20 MPa were then calculated. Multiple studies reported impact stresses greater than 20 MPa led to chondrocyte death, collagen matrix rupture, and cartilage degeneration[56,102,103,132,161,164].

Analysis of Chondrocyte Viability

Cartilage samples from control and injury joints were stained for cell viability at time of harvest. In brief, a 100-micron thick full-depth sample was prepared and incubated in PBS containing 1 μmol/L ethidium homodimer-1 and 1 μmol/L calcein AM from the Live/ Dead Viability/Cytotoxicity Kit (Invitrogen). Staining was visualized on an inverted fluorescent microscope with 495 nm/515 nm excitation/emission for calcein (live cells) and 495 nm/635 nm excitation/emission for ethidium homodimer (dead cells).

Image analysis was conducted to determine the number of live and dead cells, as described in our previous study[164]. A defined region of interest was identified (1.11mm wide x 0.88 mm deep at the center of the sample, measured from the surface down) from images taken through a 10X objective lens utilizing MATLAB R2014a (MathWorks Natick, MA). The resulting images from region of interest was analyzed with ImageJ

software, and total number of cells (number of live cells + number of dead cells) and cell viability (number of live cells x 100/ total number of cells) was calculated.

Analyses of mRNA and miRNA Expressions

Cartilage tissue adjacent to the areas used for cell viability staining was processed for analyses. For meniscus, tissue samples from impacted region were used for analyses. Synovial membrane samples were taken from the lateral and medial sides of the joint capsule. Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform extraction method[26]. For mRNA expression analyses, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize cDNA from 0.25µg total RNA following manufacturer's instructions. For miRNA expression analyses, the qScript microRNA cDNA synthesis kit (Quanta Biosciences) was used to polyadenylate miRNAs and synthesize cDNA from 0.1µg total RNA following manufacturer's instructions. Expression of selected mRNA and miRNA was analyzed using SteponePlus (Applied Biosystems) quantitative real-time PCR system. Expression of ribosomal 18S and RNU6B were used as endogenous controls for mRNA and miRNA, respectively. Primer sequences are listed in Table 2. Quantification of expression levels was determined by the $2^{-\Delta\Delta Ct}$ method and normalized to expression levels of respective control leg.

Table 2 Quantitative real-time PCR primers

Gene	Primer Sequence	Reference/ Genebank/ miRbase Accession #
hsa-miR-146a-5p	5'-TTTGAGAACTGAATTCCATGGGTTG-3'	MIMAT0000449
hsa-miR-22-5p	5'- AGTTCTTCAGTGGCAAGCTTTA-3'	MIMAT0004495
hsa-miR-34a-5p	5'-TGGCAGTGTCTTAGCTGGTTGT-3'	MIMAT0000255
hsa-miR-27b-5p	5'-AGAGCTTAGCTGATTGGTGAACA-3'	MIMAT0004588
hsa-miR-140-5p	5'- AGTGGTTTTACCCTATGGTAG-3'	MIMAT0000431
ssc-miR-125b-5p	5'- TCCCTGAGACCCTAACTTGTGA-3'	MIMAT0000423
hsa-miR-16-5p	5'-TAGCAGCACGTAAATATTGGCG-3'	MIMAT0000069
RNU6B	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′ R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′	[167]
ADAMTS-4	F:5'-AGGAGGAGATCGTGTTTCCAGAGA-3' R:5'-AAAGGCTGGCAAGCGGTACAACAA-3'	[184]
ADAMTS-5	F:5'-TTCGACATCAAGCCATGGCAACTG-3' R:5'-AAGATTTACCATTAGCCGGGCAGG-3'	[184]
IL-1β	F:5'-AATGCAACAGGGTGTGGGC-3' R:5'-CCACACCAGAAGTGCATTTG-3'	X74568.1
TNF-α	F:5'-CCCTGTGAGGGGGCAGGACA-3' R:5'-ACCCAAGGACCCCAGCGAGT-3'	X54859.1
18S	F:5'-CGGCTACCACATCCAAGGA-3' R:5'-AGCTGGAATTACCGCGGCT-3'	[184]

Immunohistochemical Analysis

Cartilage samples from impacted areas on control and injured joints were placed in 10% neutral buffered formalin for 24 hours. The samples were paraffin embedded, and sectioned cross-sectionally (5μm) for staining. Immunohistochemistry was used to localize IL-1β and TNF-α cytokine proteins within the cartilage tissue. Endogenous peroxidase was quenched for 30 minutes in the dark with 0.3% H2O2 in distilled water. After rinsing with distilled water and PBS, 10% normal horse serum was used to block nonspecific background. Sections were stained with a rabbit polyclonal antibody against human IL-1β (anti-human IL-1β (H-153), sc-7884; Santa Cruz Biotechnology, Inc) or human TNF-α (anti-human TNF-α (H-156), sc-8301; Santa Cruz Biotechnology, Inc) at 1:50 dilution at 4°C for 18 hours. Chromogenic detection was achieved with diaminobenzidine (DAB) substrate (Vectastain), followed by counterstaining with hematoxyline. Sections were observed and digitally photographed at 200x magnification.

Statistical Analysis

In this study, we analyzed 3 hour control joints (n=3), 8hr control joints (n=10), 3hr injury joints (n=3), and 8hr injury joints (n=5). From each joint, four tissue samples each were harvested from the impacted regions of the femoral condyle and meniscus. Two samples of synovial membrane were taken from the medial and lateral sides of the joint capsule. In model development, comparisons between injured area and maximum stress on the medial and lateral condyles and between control and injury groups at 3 and 8 hours were performed by two-sample two-tailed Student's t-test. For all tests, a p-value <0.05 was considered statistically significant.

2.3 Results

Model Development

The average impact load was 4.7 ± 1.6kN (n=8) as measured by the impact force sensor below the platform. After creating a standard curve (Figure 2.1), pressure distribution analysis (Figure 2.2) showed an average combined injured area of 23.5±1.9 mm^2 (n=8) greater than 20MPa on both medial and lateral condyles with the lateral and medial condyles having roughly equal injured areas (lateral: 9.6±2.7 mm^2, medial: 8.4±3.2 mm^2, p=0.75). In addition, there was no significant difference between maximum pressure recorded on medial (25.1±1.9 MPa) and lateral (25.5±1.1 MPa) condyles after impact (p=0.64).

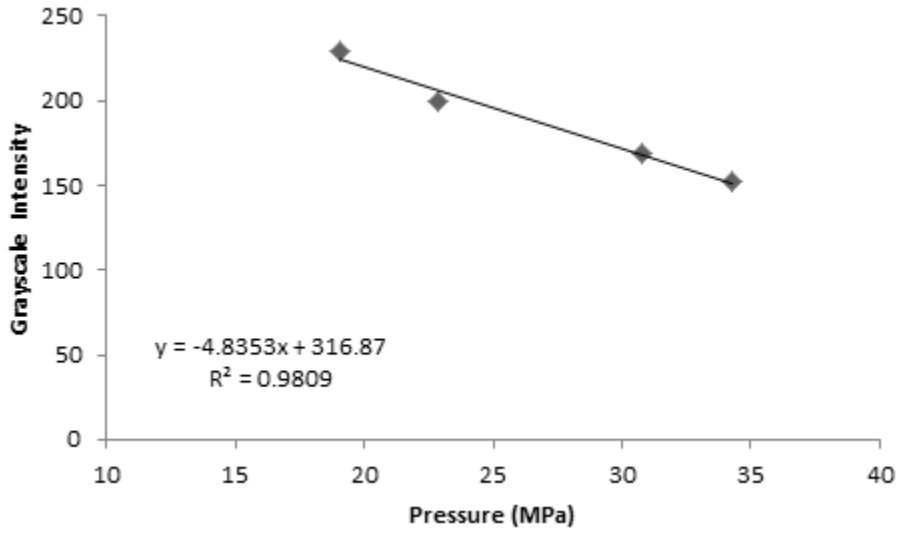

Figure 2.2 - Calibration curve showing a linear relationship between grayscale image intensity and applied pressure.

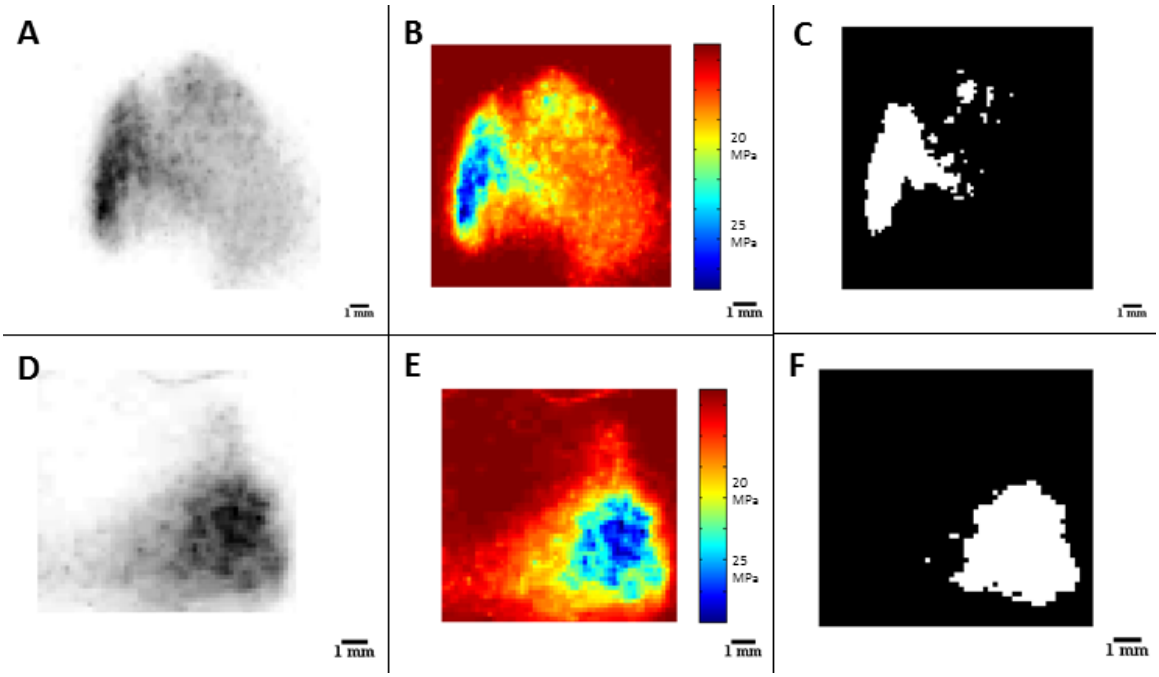

Figure 2.3- Example analysis of Fujifilm: typical medial condyle stain (A-C) and typical lateral condyle stain (D-F). A&D. Adjusted grayscale stain of digital raw stain (600ppi) with averaged intensities calculated over areas 4x4 pixel areas and applied pressure distribution map. B&E. Colormap of pressures. C&F. Region identified in white with >20 MPa applied pressure

Effect of Injury on Cell Viability and Inflammatory and Catabolic Gene Expression

At 3 hours post-injury, the injury group tended for significantly lower cell viability as compared to control (61.99±7.74% vs. 76.17±7.14%, p=0.12). At 8 hours post-injury, cell viability analysis showed significantly decreased viability in injury group as compared to control (62.3±5.9% vs. 82.2±2.5%, p=0.015) (Figure 2.3).

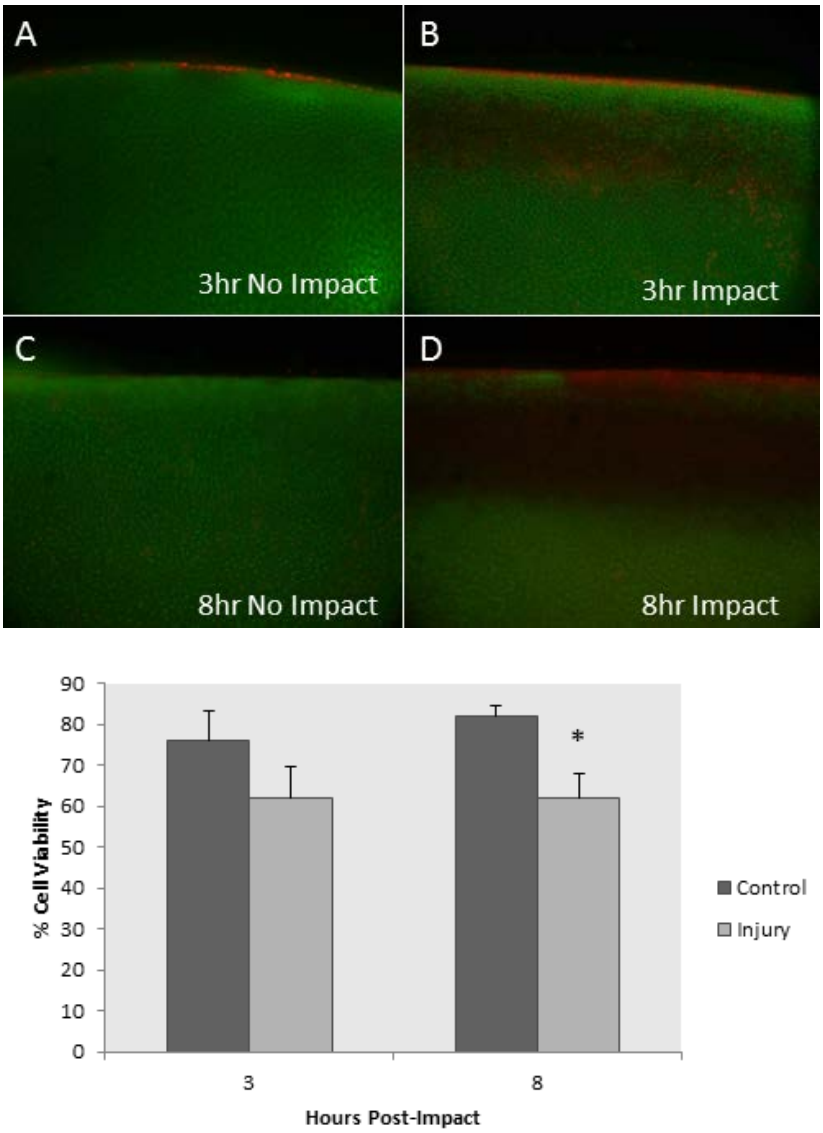

Figure 2.4 - Representative cell viability staining of cartilage at 3 (A, B) and 8 (C, D) hours post-injury(10X) Live cells (green), dead cells (red). E. Quantified cell viability of control and injured cartilage at 3 and 8 hours post-impact. Data represented as mean+SD *indicates significance p<0.05.

Analyses of mRNA expression in cartilage after injury showed an increase in ADAMTS-4 (p=0.0001) and MMP-3 (p=0.001) at 8 hours and ADAMTS-5 (p=0.05, 0.001) expression at 3 and 8 hours, respectively (Figure 2.4). IL-1β and TNF-α were significantly up-regulated at 3 and 8 hours post-impact, maintaining similar levels of up-

regulation across both time points ($p<0.01$). Increased IL-1β and TNF-α expression in injured cartilage 8 hours post-impact was verified by immunohistochemical staining (Figure 2.5).

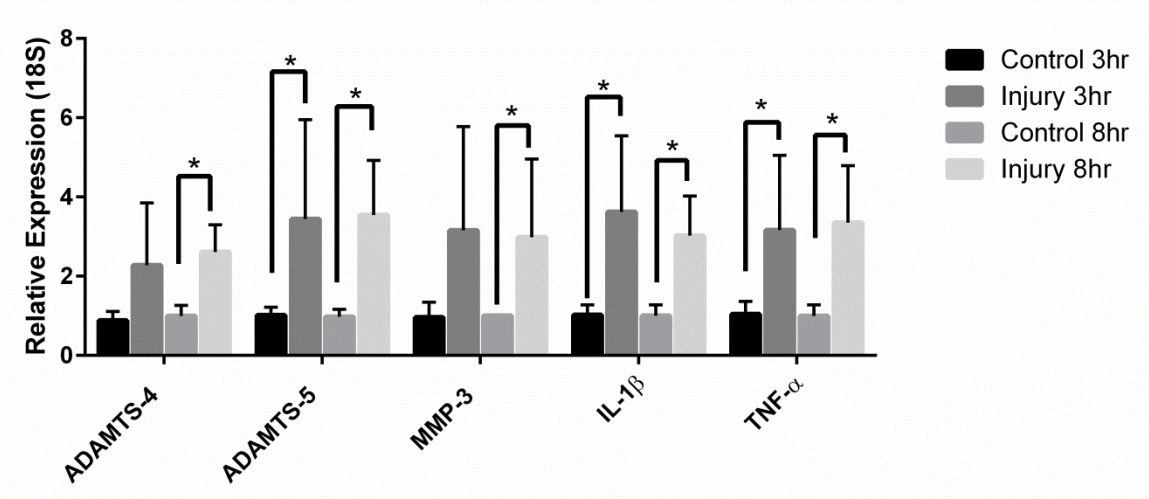

Figure 2.5 - Relative expressions of inflammatory cytokines and catabolic enzymes in cartilage at 3 and 8 hours after impact injury. *indicates significance $p<0.05$.

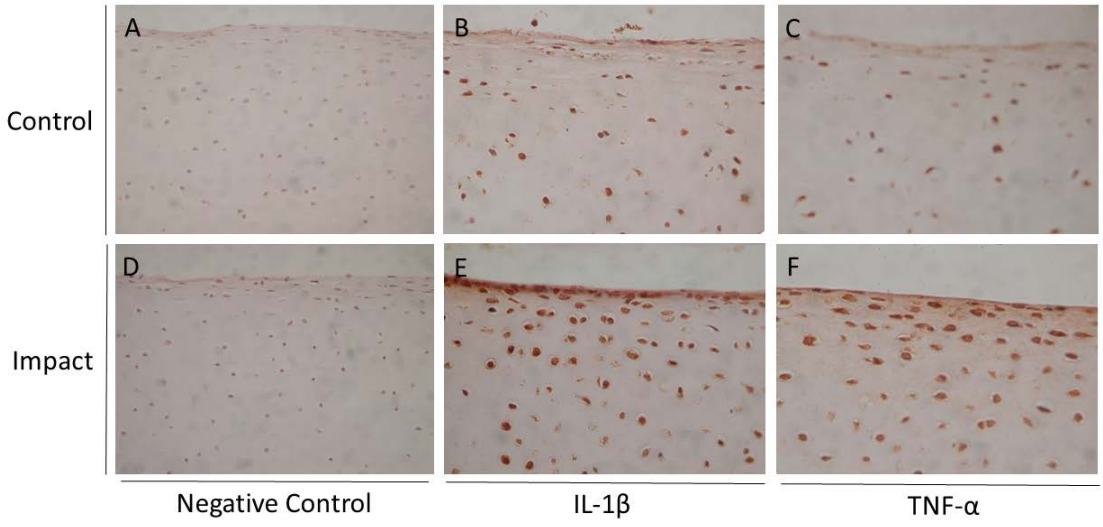

Figure 2.6 - Immunohistochemical assessments of control and injured cartilage for expressions of IL-1β (B,E) and TNF-α (C,F). Negative control sections for control (A) and injured (D) cartilage showed negligible staining. Representative samples are shown. Original magnification x 200.

Effect of Injury on miRNA Expression of Joint Tissues

At 3 hours post-injury, miRNA in cartilage are all significantly up-regulated ($p<0.05$, Table 3). At 8 hours, however, miR-125b and miR-140 were significantly down-regulated ($p=0.05$ and 0.03, respectively).

Table 3 Relative expressions of miRNAs in cartilage at 3 and 8 hours after impact injury.

	3hr Post-Impact			8hr Post-Impact		
	Mean Fold Change	**SD**	**P-value**	**Mean Fold Change**	**SD**	**P-value**
miR-125b	2.78	2.43	***0.003***	0.77	0.36	***0.05***
miR-140	5.62	5.63	***0.03***	0.73	0.54	***0.033***
miR-146a	2.68	1.85	***<0.001***	1.89	1.92	0.065
miR-22	2.39	1.69	***0.001***	0.76	0.61	0.067
miR-27b	2.05	1.89	***0.026***	1.17	1.01	0.7
miR-34a	7.76	8.12	***<0.001***	1.33	1.18	0.3481

In meniscus, all miRNAs but miR-140 were significantly down-regulated at 3 hours post-impact ($p<0.05$), but at 8 hours post-impact, only miR-22, -27b, and -34a remained significantly down-regulated ($p<0.001$, 0.003, and 0.03, respectively) (Table 4).

Table 4 Relative expressions of miRNAs in meniscus at 3 and 8 hours after impact injury.

	3hr Post-Impact			8hr Post-Impact		
	Mean Fold Change	**SD**	**P-value**	**Mean Fold Change**	**SD**	**P-value**
miR-125b	0.72	0.38	***0.029***	0.86	0.51	0.175
miR-140	1.41	0.24	0.11	1.06	0.84	0.854
miR-146a	0.53	0.30	***0.005***	1.13	0.78	0.471
miR-22	0.56	0.28	***<0.001***	0.60	0.35	***<0.001***
miR-27b	0.69	0.39	***0.04***	0.61	0.43	***0.003***
miR-34a	0.63	0.19	***0.009***	0.74	0.51	***0.033***

In synovial membrane, miR-125b, -140, -146a, and -27b were significantly up-regulated at 3 hours post-impact (p=0.01, 0.02, 0.01, and 0.01, respectively), and at 8 hours post-impact, all miRNA except miR-125b were significantly down-regulated ($p<0.05$) (Table 5).

Table 5 Relative expressions of miRNAs in synovial membrane at 3 and 8 hours after impact injury.

	3hr Post-Impact			8hr Post-Impact		
	Mean Fold Change	**SD**	**P-value**	**Mean Fold Change**	**SD**	**P-value**
miR-125b	3.98	4.16	***0.010***	1.02	0.85	0.972
miR-140	1.55	0.36	***0.020***	0.62	0.57	***0.043***
miR-146a	5.28	6.02	***0.013***	0.58	0.26	***0.017***
miR-22	1.73	1.22	0.083	0.64	0.47	***0.027***
miR-27b	3.11	2.98	***0.012***	0.55	0.36	***0.004***
miR-34a	1.29	0.66	0.138	0.61	0.57	***0.039***

2.4 Discussion

The *ex-vivo* intact joint impact injury model developed in this study presents a method to examine the acute phase of impact injury and can be used to investigate the efficacy of potential treatments. A detailed understanding of the events that occur during the acute phase injury in cartilage can provide great insight or help choose suitable time points, targets, and methods of interventional treatment to prevent or delay the progression of PTOA. This is the first study to report differential miRNA expressions in cartilage, meniscus, and synovial membrane after impact injury using the *ex-vivo* intact joint impact injury model.

In normal joint loading *in-vivo*, the force is distributed 70% to medial condyle and 30% to the lateral condyle[109,182]. However, even though the forces are unequally distributed, the pressures remain similar due to the larger contact area and cartilage

thickness of the medial condyle[159]. Based on the Fujifilm data, it was found that both femoral condyles were injured simultaneously while similar maximum contact stresses on the condyles were observed, showing that a physiological distribution of stresses on the articular cartilage was effectively created in the *ex-vivo* impact injury model using an intact joint. Furthermore, the contact stresses, greater than 20MPa, covered a total combined area of 23.5±1.9 mm^2 of cartilage. The impact forces applied and pressures recorded are within the range of deleterious cartilage impact as pressures of 20MPa or greater have been reported to lead to critical cell death, collagen matrix rupture, and eventual cartilage degeneration in several long term *in-vitro* and *in-vivo* studies[56,102,132,161]. It can be inferred that the physiologically relevant acute injury response of cartilage is accurately represented in this model, as confirmed by viability staining of injured area. The *ex-vivo* injury model has also proven consistent with *in-vivo* models of acute cartilage injury that reported peak IL-1β expression within 4 hours of injury in an *in-vivo* murine model[80].

Upon the comparison of the control and injury groups, a significant decrease in cell viability was seen between non-injured control and injury groups. The observed decrease in viability and region of cell death is consistent with *in-vitro* explant models[103,124,161] and clinical studies[51]. The initial mechanism of cell death in the superficial zone of cartilage after impact injury is hypothesized to result from excessive mechanical strain on the tissue and cells at the cartilage surface[44]. Another possible mechanism of cell death is cellular apoptosis caused by high concentrations of IL-1β[81,143].

Inflammation in the cartilage and knee joint, driven by IL-1β and TNF-α[60,149], plays a crucial role in the development of OA. High mechanical stress on chondrocytes

activates the IL-1 pathway and results in the secretion of IL-1β[111,149]. It is well understood that exogenous IL-1β secreted by surrounding knee tissues incites a cascading effect on the catabolic responses of cartilage[60]. In this study, IL-1β and TNF-α are significantly up-regulated at 3 hours and remain up-regulated at 8 hours after impact injury as shown by gene expression analysis and immunohistochemisty. Concurrently, mechanical injury incites transient up-regulation of all miRNAs investigated and ADAMTS-5 at 3 hours post-impact. However, at 8 hours, secreted IL-1β binds to the IL-1 receptor, which incites the IL-1 catabolic pathway, and then down-regulates miR-140 and miR-125b. Down-regulation of miR-125b and miR-140 has been directly linked to elevated expression levels of ADAMTS-4 and ADAMTS-5, respectively[96,106]. As a result, elevated aggrecanase and inflammatory cytokine expressions remain at 8 hours post-injury.

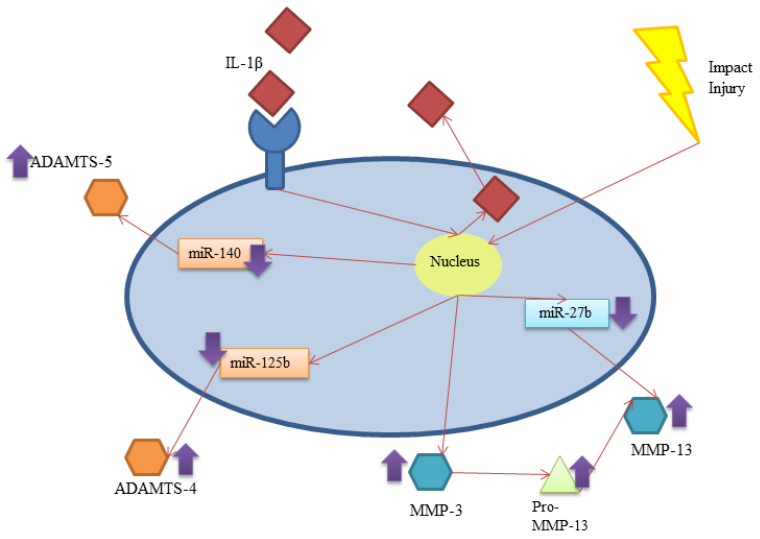

Figure 2.7 - Proposed Mechanism of Injury on catabolic regulation at 8 hours. Up-regulation or down-regulation of a specific gene is represented by an upward or downward arrow, respectively. All listed targets have been validated. The miRNA targets listed include aggrecanase-1 (ADAMTS-4), aggrecanase-2 (ADAMTS-5), and collagenase-3 (MMP-13).

In the *ex-vivo* injury model, the meniscus was also injured in a physiologically compressive manner. Similar to cartilage, impact injury to the meniscus caused transient miR-27b remained down-regulated. Down-regulation of miR-27b has been directly linked to elevated expression levels of MMP-13[4]. Although articular cartilage and meniscus are primarily made of chondrocytes, the two tissues distribute loads differently under impact. Different distributed loads, along with inherent genetic and material properties[118], could the differences in the miRNA responses after impact from cartilage and meniscus tissues. Lastly, because the synovial membrane is assumed to be "uninjured" in this impact model, we believe that the up-regulation and then down-regulation of miRNAs over time is a transient response in reaction to the changing contents of the synovial fluid and blood supply.

The model utilized in this study presents the ability to monitor the acute events after injury while maintaining the physiological environment of the knee joint tissues. A possible limitation of this model is the inability to perform long-term studies. While cartilage tissue remains viable for over 24 hours after donor death[6], meniscus and synovial tissue viability could decrease without renewed blood supply.

In summary, this study demonstrates that impact injury causes aberrant regulation of degenerative enzymes and inflammatory cytokines in cartilage, along with a decrease in chondrocyte viability, which may increase the risk of PTOA development. The *ex-vivo* injury model developed in this study offers a suitable method for preliminary *in-vitro* testing of early intervention therapies administered immediately after injury in a cost-effective manner prior to evaluation of the therapy *in-vivo*.

Chapter 3
Early Intervention Strategies for Acute Cartilage Injury: A Comparative Treatment Study

3.1 Background

Knee injury is the leading cause of post-traumatic osteoarthritis (PTOA) in young people and can occur as early as 10 years following a traumatic injury[22]. PTOA accounts for up to 5.6 million per year, or 12%, of the total cases of symptomatic OA in the United States[22]. Further, the incidence of PTOA is expected to increase as the number of traumatic injuries affecting young adults rises from increased rates of participation in sports and tendency towards heavier players[129]. Current therapy for PTOA is similar to that of primary OA, with optimal management requiring a combination of non-pharmacological and pharmacological modalities[181]. The limitation of these modalities is that they are for management of the disease once it has already occurred and fail to correct the underlying pathology[31], resulting in continued disease progression. Further, unlike primary OA, PTOA has a known starting point of disease, namely the injury event, and thus uniquely represents an appealing opportunity for the use of targeted therapy that could prevent or delay the onset of PTOA.

To elucidate the pathogenesis of PTOA following joint injury, we developed an ex-vivo model for acute joint injury that applies deleterious loading to the cartilage and meniscus in a physiological manner while keeping the joint capsule intact (Chapter 2). In this previous chapter, we observed increased production of IL-1β and TNF-α at 8 hours post-injury. Acute cartilage impact was also shown to decrease miR-125b and miR-140 expressions at 8 hours with concurrent increased expressions of their target genes, ADAMTS-4 and ADAMTS-5, respectively. An increased understanding of the molecular

processes involved in the pathogenesis of PTOA will provide potential targets for preventative agents. Intervention for PTOA should inhibit inflammatory responses, prevent cell death, prevent cartilage matrix degradation, and promote production of new matrix. Currently, Mesenchymal Stem Cells (MSCs)[19], Interleukin Receptor Antagonist Protein (IRAP)[72], Dexamethasone (DEX)[147,148], and hyaluronan (HA)[116] are clinically used treatments for the management of pain and inflammation in OA patients.

MSCs have been shown to exhibit immunosuppressive and chondroprotective characteristics in the clinical and preclinical treatment of OA[19]. Intra-articular injection of MSCs at the early time point of 7 days protected against synovitis and cartilage destruction in an *in-vivo* murine OA model[138,158]. Additionally, MSC intra-articular injection in multiple large animal OA models resulted in greater repaired cartilage area and reduced joint inflammation[5,76,98,114,137].

IRAP is a naturally-occurring anti-inflammatory cytokine in the body which prevents IL-1α/β from binding to its receptor and triggering its catabolic pathway[99]. Previous clinical and preclinical studies[60,67,72,99,111] have shown that intra-articular injection of IRAP reduces catabolic activities and joint discomfort in established OA. Recent studies using a mouse articular cartilage fracture model have shown that IRAP, as an early intervention treatment, significantly reduced cartilage degeneration and synovial inflammation[41,68].

DEX is a potent corticosteroid known for its anti-inflammatory properties[21,65]. Intra-articular injections of DEX have been commonly and effectively used clinically for symptomatic relief in OA patients[14,147]. More recently, several studies have shown that DEX reduces glycosaminoglycan loss in cartilage injury in vitro, enhances progenitor

cells to differentiate into chondrogenic cells and synthesize proteoglycans, and prevents aggrecan degradation[85,93,94,151]. Further, DEX decreased joint inflammation and tissue degradation in an in vivo PTOA model in rabbits [55].

HA is the principle glycosaminoglycan found in synovial fluid and is responsible for viscosity and lubrication within the joint[139,140]. Intra-articular HA therapy has been used clinically as an alternative for the treatment of knee OA pain with beneficial anti-inflammatory and chondroprotective effects[116]. HA is theorized to elicit its therapeutic anti-inflammatory chondroprotective effects primarily by stimulating more endogenous production of hyaluronate and enhancing synovial fluid viscosity or viscosupplementation[10]. Alternatively, administered HA has been suggested to prevent the degradation of endogenous HA by removing catabolic enzymes and cytokines[35].

In this study, four potential early intervention treatments for PTOA (IRAP, DEX, HA, and MSCs) were compared, and their effects on the early inflammatory and catabolic events after acute cartilage injury were determined at 8 hours after intact joint impact. We hypothesize that intra-articular treatment immediately after impact will inhibit the early inflammatory and catabolic genetic changes in acute cartilage and meniscus injury.

3.2 Materials and Methods

Injury and Administration of Treatment

The procedure described previously was used to injure the joint (Chapter 2.2). One hour after impact, 20µg of recombinant human IRAP (Peprotech; Rocky Hill, NJ), 15mg (MW 1.5-2.2 MDa) sodium hyaluronate (HA; Sigma), P3-P5 $5x10^6$ MSCs, or 4mg DEX (Sigma) in 1 mL of Phosphate Buffered Saline (PBS) was administered to injured

knee via intra-articular injection. The dose of IRAP was calculated from successful reports of inhibition of catabolic gene expression with IRAP in porcine knee tissues *in-vitro*[67]. Dosages of DEX and high molecular weight HA were chosen based on the current clinical preparations used for the treatment of OA symptoms[116,148]. The concentration of MSCs ($5x10^6$ cells/joint) used was based on that of a previous experimental OA study[158]. The contralateral joint of the same pig served as the control for each treatment and were administered 1mL PBS in the same manner previously described. At 8 hours after injury, samples from impacted region on articular cartilage on femoral condyles and meniscus from both groups were harvested, flash-frozen, and stored at -80°C for analyses of miRNA and mRNA expressions.

Analysis of Chondrocyte Viability

Cartilage samples from control and treatment joints were stained for cell viability at time of harvest as previously described (Chapter 2.2).

Analyses of mRNA and miRNA Expressions

Cartilage tissue adjacent to the areas used for cell viability staining was processed for analyses. For meniscus, tissue samples from impacted region were used for analyses. Total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform extraction method[26]. For mRNA expression analyses, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize cDNA from 0.25µg total RNA following manufacturer's instructions. For miRNA expression analyses, the qScript microRNA cDNA synthesis kit (Quanta Biosciences) was used to polyadenylate miRNAs and synthesize cDNA from 0.1µg total RNA following manufacturer's instructions. Expressions of ADAMTS-4, ADAMTS-5, MMP-3, IL-1β, TNF-α, miR-140, miR-125b,

and miR-27b were analyzed using SteponePlus (Applied Biosystems) quantitative real-time PCR system. Expression of ribosomal 18S and RNU6B were used as endogenous controls for mRNA and miRNA, respectively. Primer sequences are listed in Table 2. Quantification of expression levels was determined by the $2^{-\Delta\Delta Ct}$ method and normalized to expression levels of respective control leg.

Statistical Analysis

In this study, we analyzed 25 control joints, 5 injury without treatment joints, 5 IRAP treatment joints, 5 MSC treatment joints, 5 DEX treatment joints, and 5 HA treatment joints. From each joint, four tissue samples each were harvested from the impacted regions of the femoral condyle and meniscus. Comparison of control, injury, and treatment groups at 8 hours post-injury was performed by a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (Graphpad Prism 6). Data represented as mean+ standard error (SE). p-value <0.05 was considered significant.

3.3 Results

Chondrocyte viability after impact injury and treatment

At 8 hours post-injury and treatment, cell viability analysis showed significantly decreased viability in injury group as compared to control (ANOVA p=0.0344) (Figure 3.1). There were no significant differences found between control and any treatments nor between injury and treatments.

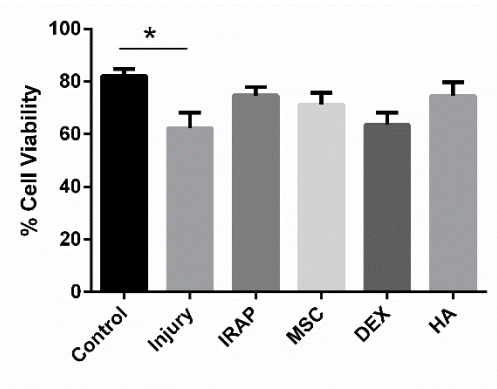

Figure 3.1 - Quantified cell viability of cartilage at 8 hours post-impact and treatment. *indicates significance p<0.05.

Effect of Injury and Treatment on Gene and miRNA Expression

IRAP and DEX treatment administered one hour after impact injury significantly prevented up-regulation of aggrecanases and inflammatory cytokines in cartilage (Figure 3.2). Expressions of IL-1β and TNF-α in cartilage were significantly decreased in IRAP and DEX-treated joints as compared to injury (both ANOVA $p<0.001$) (Figure 3.2A & B). Expression of ADAMTS-4 was significantly lower in IRAP and DEX-treated joints as compared to injury joints (ANOVA $p<0.001$) (Figure 3.2C). Expressions of ADAMTS-5 and MMP-3 were significantly lower in DEX- and IRAP-treated cartilage as compared to injury and MSC groups (ANOVA both $p<0.001$) (Figure 3.2D & E). MSC and HA administered one hour after impact injury did not significantly alter aggrecanase and inflammatory cytokine expressions as compared to the injury group.

Expression of miR-140 in cartilage was significantly up-regulated after IRAP treatment as compared to control, injury, MSC, and DEX groups (ANOVA $p<0.001$) (Figure 3.3A). After HA treatment, miR-140 expression was also significantly up-regulated as compared to control and injury. Expressions of miR-125b and miR-27b were significantly up-regulated after IRAP and HA treatment as compared to control, injury,

and MSC groups (both ANOVA p<0.001) (Figure 3.3B & C). IRAP and HA treatments significantly up-regulated miR-22 expression as compared to control, injury, and DEX groups (ANOVA p<0.001) (Figure 3.3D). Expression of miR-146a was significantly increased after IRAP and HA treatments as compared to control (ANOVA p=0.002) (Figure 3.3E). Expression of miR-34a was significantly up-regulated by HA treatment as compared to control, injury, and DEX groups (ANOVA p<0.001) (Figure 3.3G). No significant difference in miRNA expressions was found among MSC and DEX groups as compared to the control and injury groups. There was no significant difference in miR-16 expression in cartilage among groups.

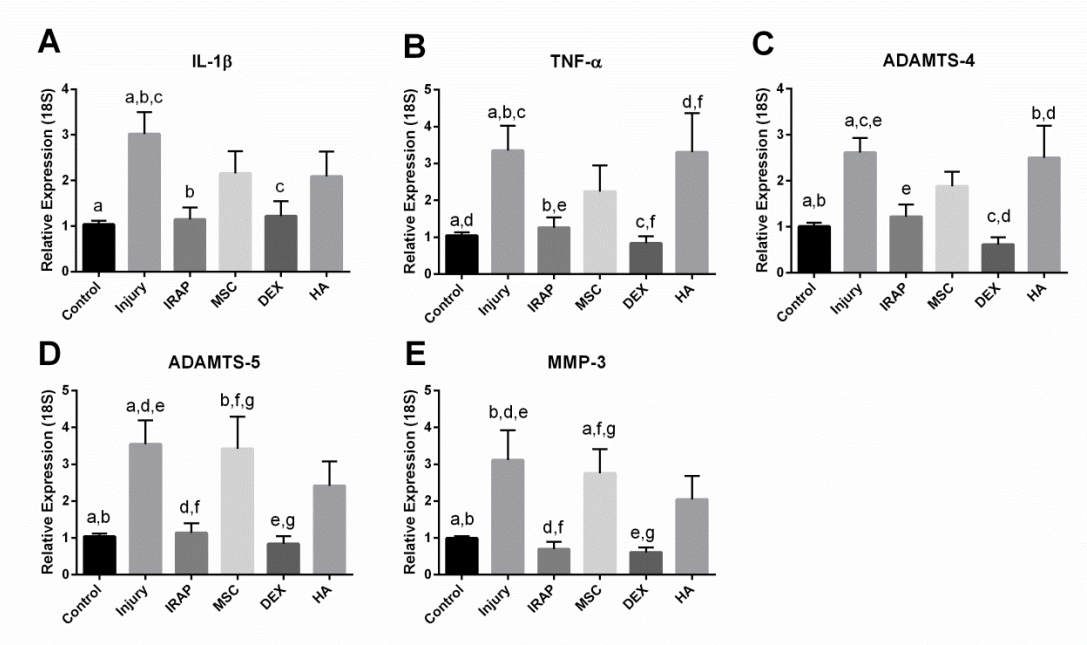

Figure 3.2 - Relative expressions of (A) IL-1β, (B) TNF-α, (C) ADAMTS-4, (D) ADAMTS-5, and (E) MMP-3 in cartilage at 8 hours after impact injury and treatment. Groups with the same letter are significantly different from each other (p<0.05).

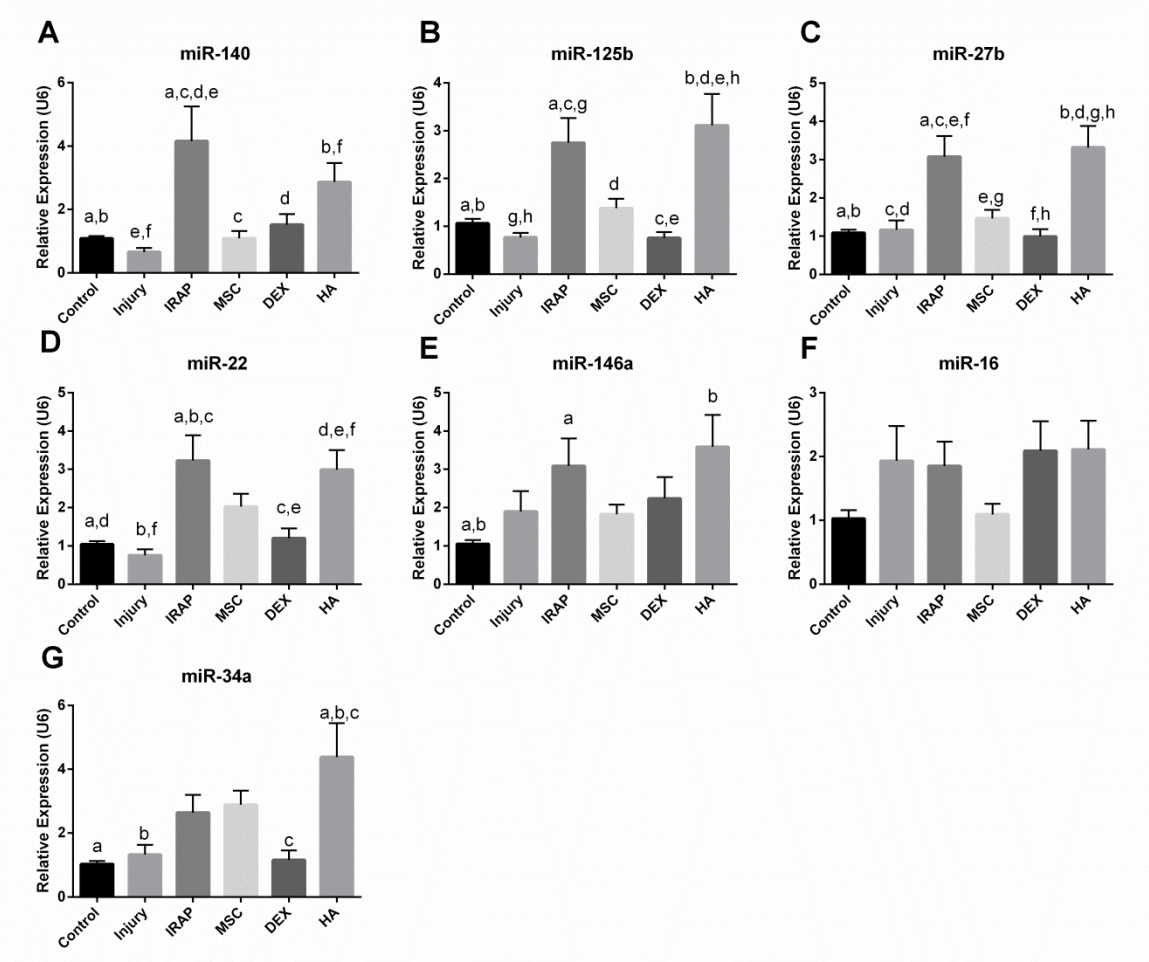

Figure 3.3 - Relative expressions of (A) miR-140, (B) miR-125b, (C) miR-27b, (D) miR-22, (E) miR-146a, (F) miR-16, and (G) miR-34a in cartilage at 8 hours after impact injury and treatment. Groups with the same letter are significantly different from each other ($p<0.05$).

Because this intact joint model allows for simultaneous treatment of intra-articular tissues, we examined the mRNA and miRNA expressions in meniscus 8 hours after injury and treatment (Figure 3.4 and Figure 3.5). After impact injury and no treatment, expressions of MMP-3, IL-1β, and TNF-α were significantly up-regulated in meniscus tissue (ANOVA $p=0.002$, <0.001, and 0.002, respectively) (Figure 3.4A, B, &E). However, when IRAP, HA, and DEX were administered one hour after injury, expressions of IL-1β and MMP-3 in meniscus were no longer significantly different from

the control group and were significantly lower than the injury group. Expression of TNF-α was also significantly lower than the injury group in IRAP- and HA-treated meniscus.

Expression of miR-140 in meniscus was significantly increased as compared to control, injury, IRAP, and MSC groups after DEX and HA treatments (ANOVA $p<0.001$) (Figure 3.5A). HA significantly increased miR-125b expression in injured meniscus as compared to control, injury, DEX, and MSC groups (ANOVA $p<0.001$) (Figure 3.5B). After IRAP and MSC treatment, miR-27b was significantly increased in meniscus as compared to control, injury, and MSC groups (ANOVA $p<0.001$) (Figure 3.5C). Expressions of miR-22 and miR-16 were significantly up-regulated after HA treatment as compared to control and injury groups (ANOVA both $p<0.001$) (Figure 3.5D & F). Expressions of miR-146a and miR-34a were significantly up-regulated after HA treatment as compared to control and injury groups (ANOVA both $p<0.002$) (Figure 3.5E & G). Expression of miR-146a was also significantly higher in HA treated meniscus than in IRAP- and MSC-treated meniscus.

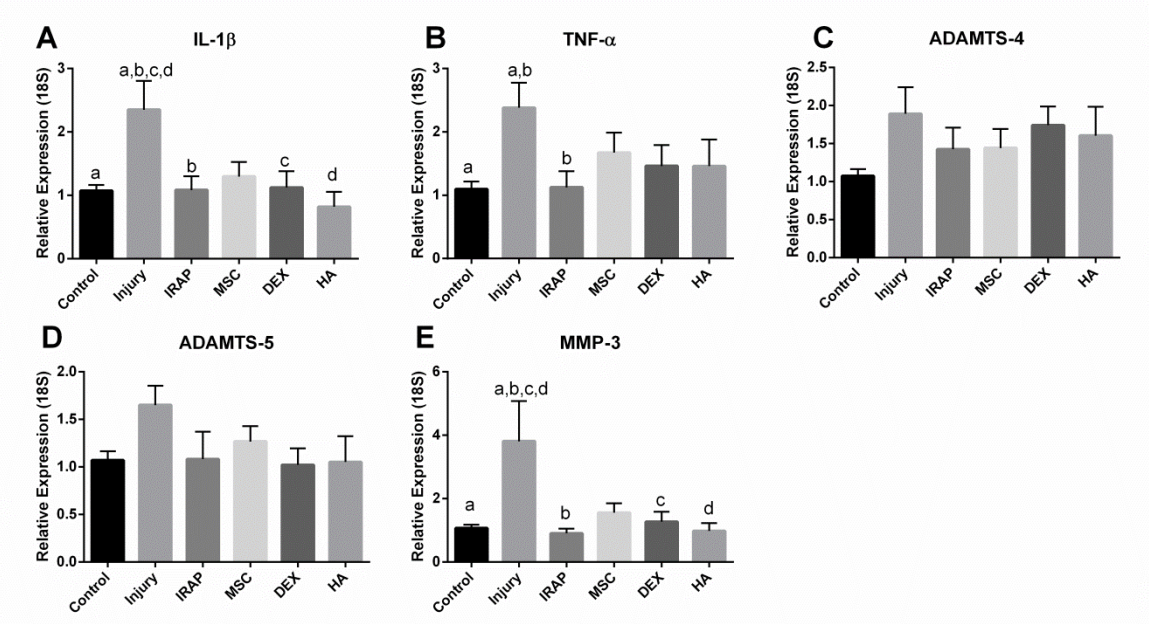

Figure 3.4 - Relative expressions of (A) IL-1β, (B) TNF-α, (C) ADAMTS-4, (D) ADAMTS-5, and (E) MMP-3 in meniscus at 8 hours after impact injury and treatment. Groups with the same letter are significantly different from each other ($p<0.05$).

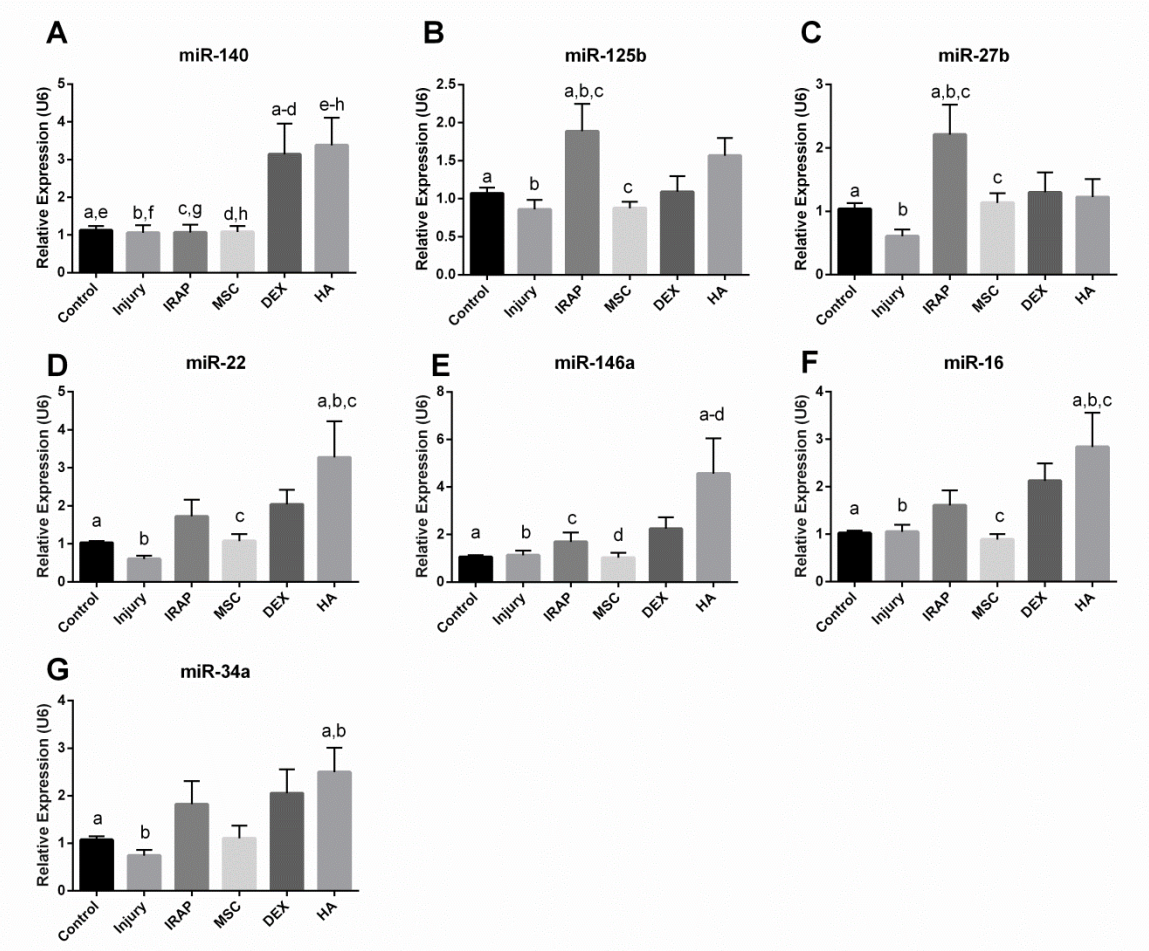

Figure 3.5 - Relative expressions of (A) miR-140, (B) miR-125b, (C) miR-27b, (D) miR-22, (E) miR-146a, (F) miR-16, and (G) miR-34a in meniscus at 8 hours after impact injury and treatment. Groups with the same letter are significantly different from each other ($p<0.05$).

3.4 Discussion

The results of this study support the hypothesis by demonstrating that therapeutic early intervention during the acute phase of injury can significantly alter the genetic injury response in injured cartilage. This is the first study to report differential miRNA expressions in cartilage after impact injury and intra-articular treatment on miRNA expression using the *ex-vivo* intact joint impact injury model.

Inflammation in the cartilage and knee joint, driven by IL-1β and TNF-α[60,149], plays a crucial role in the development of OA. High mechanical stress on chondrocytes

activates the IL-1 pathway and results in the secretion of IL-1β[111,149]. It is well understood that exogenous IL-1β secreted by surrounding knee tissues incites a cascading effect on the catabolic responses of cartilage[60]. In this study, IL-1β and TNF-α are significantly up-regulated at 3 hours and remain up-regulated at 8 hours after impact injury as shown by gene expression analysis and immunohistochemisty. Concurrently, mechanical injury incites transient up-regulation of miR-125b, miR-140, and ADAMTS-5 at 3 hours post-impact (Chapter 2). However, at 8 hours, secreted IL-1β binds to the IL-1 receptor, which incites the IL-1 catabolic pathway, and then down-regulates miR-140 and miR-125b. As a result, elevated aggrecanase and inflammatory cytokine expressions remain at 8 hours post-injury. Nevertheless, after intra-articular injection of IRAP given one hour after impact, IL-1β and TNF-α were no longer up-regulated at 8 hours. Administration of IRAP serves to antagonize the catabolic cascade (Figure 3.6) by preventing IL-1β from binding to the IL-1 receptor on the cell membrane, and inhibits the paracrine and autocrine effects of IL-1β secreted due to mechanical loading[99]. Therefore, expressions of miR-140 and miR-125b remain elevated and expressions of aggrecanases and inflammatory cytokines are down-regulated. Additionally, IRAP treatment up-regulated miR-146a expression which may be an attempt to reduce the production of IL-1β and TNF-a receptors IRAK1 and TRAF6[81,177]. Previous cell culture studies have shown that overexpression of miR-146a in IL-1β stimulated chondrocytes down-regulated inflammatory and catabolic activity[81,177]. Although up-regulation of miR-22 has been associated with an up-regulation of IL-1β[57], this was not observed in cartilage after IRAP treatment. MiR-22 has multiple validated targets (e.g. PPARA and BMP-7)[57] that may have been affected by the up-regulation of miR-22 by IRAP treatment. In-vivo

studies are still required to determine the long-term effects of the up-regulation of miR-22 by IRAP treatment.

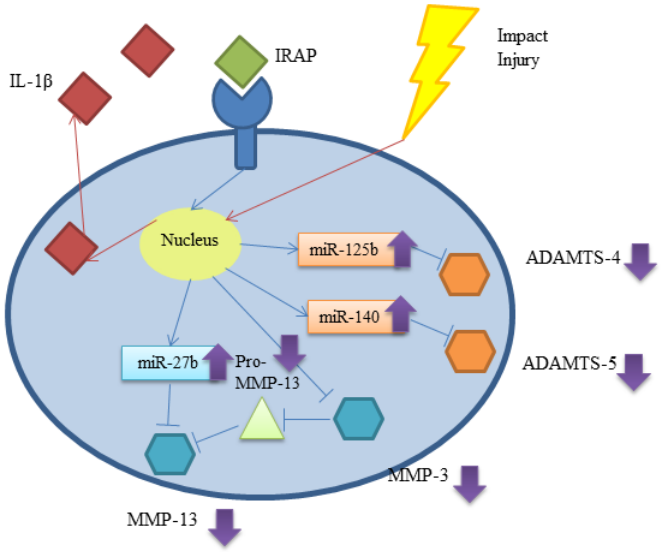

Figure 3.6 - Proposed Mechanism of IRAP Treatment on catabolic regulation at 8 hours. Up-regulation or down-regulation of a specific gene is represented by an upward or downward arrow, respectively. All listed targets have been validated. The miRNA targets listed include aggrecanase-1 (ADAMTS-4), aggrecanase-2 (ADAMTS-5), and collagenase-3 (MMP-13).

As a corticosteroid, DEX passes freely through the cell and nuclear membrane and binds to an intracellular glucocorticoid receptor which directly inhibits the up-regulation of inflammatory cytokines at the transcriptional level[21,65]. This action was observed in DEX-treated cartilage as the up-regulation of aggrecanases and inflammatory cytokines was inhibited without modulating expressions of the miRNAs examined (Figure 3.7). The inhibition of degenerative enzyme expressions after IRAP and DEX treatment greatly support the potential of early intervention to prevent cartilage degeneration. Nonetheless, the evidence of inhibition of catabolic gene expression in acutely injured cartilage after treatment should be considered preliminary; therefore, these findings need to be confirmed and expanded upon in longitudinal *in-vivo* studies.

Additional studies with varying doses of treatments administered after injury are needed to determine optimal doses for long-term effect *in-vivo*.

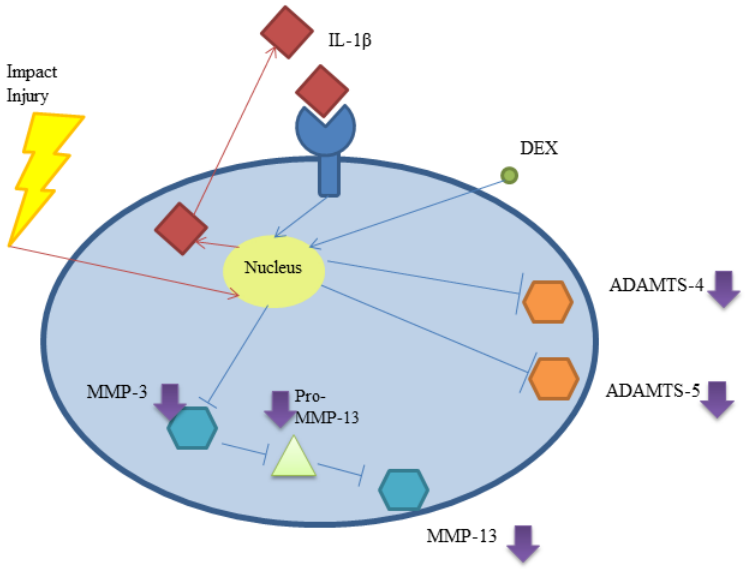

Figure 3.7 - Proposed mechanism of DEX treatment on catabolic regulation at 8 hours. Up-regulation or down-regulation of a specific gene is represented by an upward or downward arrow, respectively.

Intra-articular injection of high molecular weight (MW) HA (>1 x 10^6 Da) is widely accepted as beneficial for viscosupplementation in OA joints or acutely injured cartilage. HA also has been shown to elicit a cellular response by binding to specific receptors, such as cluster determinant (CD)-44 and receptor for hyaluronate-mediated motility (RHAMM), that are expressed by many cells including synoviocytes and chondrocytes[36,58]. Studies have shown that high MW HA suppresses synovial membrane IL-1β production by acting on the synovial CD-44 receptor[157]. In chondrocytes, it is theorized that high MW HA binds to the CD-44:RHAMM complex and prevents receptor cross-linking and adverse cellular effects, such as activation of the nuclear factor (NF)-κβ pathway[100]. In this study, at 8 hours after injury, HA had no effect on the aggrecanase and inflammatory cytokine expression in cartilage, which may indicate that the therapeutic

effects of HA via suppression of synovial IL-1β may not be observed before 8 hours. However, HA significantly up-regulated miR-125b miR-27b, mir-146a, and miR-140 expressions in cartilage, suggesting an attempt to down-regulate the increased catabolic activity after injury[96,106], possibly through the cartilage CD-44 receptors (Figure 3.8). Additionally, HA treatment up-regulated miR-22 expression, similar to IRAP treatment, but was also accompanied by a tendency for up-regulation of IL-1β. Up-regulation of miR-22 by HA treatment may be promoting the up-regulation of IL-1β in cartilage[57]. HA treatment also up-regulated miR-22 and miR-34a expressions which have been associated with an increase in IL-1β production[57] and apoptotic activity[1,117]. While no significant decrease in cell viability was observed after HA treatment at 8 hours, the apoptotic effect of miR-34a on cartilage viability may be observed at a longer time point. Further in-vitro and in-vivo studies to investigate the mechanism of HA treatment and its targets are needed.

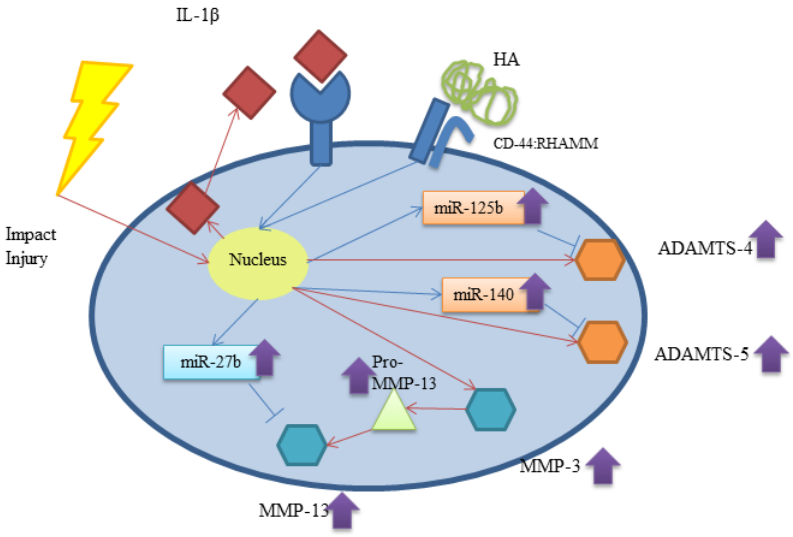

Figure 3.8 - Proposed mechanism of HA treatment on catabolic regulation at 8 hours. Up-regulation or down-regulation of a specific gene is represented by an upward or downward arrow, respectively. All listed targets have been validated. The miRNA targets listed include aggrecanase-1 (ADAMTS-4), aggrecanase-2 (ADAMTS-5), and collagenase-3 (MMP-13).

Previous in-vivo studies have reported that the beneficial effects of MSC treatment after cartilage injury are due to the production and release of anti-inflammatory cytokines, seen as early as day 2 of treatment[136,155]. The null effect of MSCs observed in this study may be due to number of cells used or the time point of 8 hours is too short for the MSCs to produce anti-inflammatory proteins at an effective concentration.

Intra-articularly injected HA significantly up-regulated miR-140, miR-146a, miR-22 and miR-34a in meniscus, similar to what was seen in cartilage. HA treatment also up-regulated expression of miR-16, which directly regulates BCL-2, a potent apoptosis inhibitor[27]. Similar to cartilage, the apoptotic effects of miR-34a and miR-16 dysregulation may affect the cell viability at a time point greater than 8 hours. IRAP treatment up-regulated miR-27b expression which may indicate increased inhibition of collagenase after treatment[4]. However, all treatments when administered 1 hour after injury tended to have lower inflammatory cytokine and MMP-3 expressions at 8 hours. It is known that meniscus and cartilage distribute loads differently under impact due to inherent genetic and material property differences[118]. Therefore, differing material properties could result in differing mechanobiological responses from cartilage and meniscus tissues. Because the injury on the meniscus was less severe than on cartilage, as shown by the lower relative expressions of inflammatory cytokines and aggrecanases, the doses of the respective treatments may have been more effective in inhibiting the up-regulation of IL-1β, TNF-α, and MMP-3.

Upon the comparison of the control, injury, and treatment groups, a significant decrease in cell viability was seen between non-injured control and non-treated injury groups. In our study, there was no significant difference between any of the treatment

groups and the control group or between any of the treatment groups and the injury group. While most of the superficial layer chondrocytes would have died by necrosis from the excessive strain induced by impact[44], our findings suggest that the 4 early intervention therapies administered one hour after impact prevented further cell death as compared to the non-treated injury group.

The rationale for administering treatment at 1 hour after injury was based on the need to demonstrate proof of concept but probably not mimic a clinically relevant situation in the health care system where access to care is limited. The model utilized in this study presents the ability to monitor the acute events after injury while maintaining the physiological environment of the knee joint tissues. A possible limitation of this model is the inability to perform long-term studies. While cartilage tissue remains viable for more than 24 hours after death of the donor due to its avascular nature[6], meniscus and synovial membrane tissue viability would substantially decrease due to the lack of nutrients. However, the *ex-vivo* injury model developed in this study provides the opportunity for preliminary *in-vitro* testing of early intervention therapies administered immediately after injury in a cost-effective manner prior to evaluation of the therapy *in-vivo*.

In summary, this study demonstrates that early intervention with molecular treatment administered during acute phase of cartilage impact injury modulates the catabolic microRNA and gene expression in cartilage and meniscus via different mechanisms. The aberrant regulation of degenerative enzymes and inflammatory cytokines in cartilage, along with a decrease in chondrocyte viability, after impact injury

increases the risk of PTOA development. Clinically, these findings support the potential of early intervention strategies for the prevention of cartilage degeneration after impact injury.

Chapter 4
Inflammatory Cytokines Induce Time- and Concentration-Dependent MicroRNA Expression in Chondrocyte Media: Relevance to Osteoarthritis

4.1 Background

Osteoarthritis (OA) is the most prevalent joint disease in the United States, affecting more than 27 million Americans[75]. Currently, there are no reliable methods of diagnosing OA before radiological evidence of the disease develops, which hinders the effectiveness of therapies and potential preventative measures. Several studies have investigated the expression of molecular by-products of cartilage degeneration, such as cytokines and matrix fragments, as potential biomarkers of OA[24,104,152]. However, these techniques require the degeneration process to have begun, resulting in irreversible cartilage damage already at the time of diagnosis.

MicroRNAs (miRNAs) are a type of short non-coding RNA that regulate the expression of various genes post-transcriptionally[13,77]. In brief, miRNAs negatively regulate target messenger RNA (mRNA) post-transcriptionally by promoting degradation, deadenylation, or repression of translation[12]. MiRNAs have been shown to be released into the serum and synovial fluid in a quite stable form, and that miRNAs released from cells may be by-products of necrotic or apoptotic cells during disease[110,162,180]. With the discovery of stable miRNAs in both tissues and body fluids, a great number of publications have already described certain miRNAs as biomarkers of breast, colon, gastric, lung, oral, ovarian, pancreatic, prostate, tongue, and squamous cell cancers (Review; Brase et al.[20]). Therefore, differential expressions of miRNAs in the synovial fluid may be suitable as potential non-invasive biomarkers of OA progression.

The miRNAs found in the synovial fluid are comprised of miRNAs not only from articular cartilage but also from the meniscus and synovial membrane. Thus, in order to successfully diagnose OA, the miRNA biomarker must be distinctly released by cartilage when in the presence of inflammatory cytokines characteristic of early OA, Interleukin (IL)-1β and Tumor Necrosis Factor (TNF)-α[43,60]. In this study, we examined the cellular and extracellular expressions of five candidate miRNAs in chondrocytes, synoviocytes, and meniscus cells after simulated injury with IL-1β and TNF-α. The miRNAs of interest have been evaluated in the literature as genetic markers of cartilage degeneration (miR-140[106], miR-27b[4]), apoptosis (miR-16[27]), and inflammation (miR-22[57], miR-146a[177]) in developed OA or its symptoms. MiR-27b directly regulates the expression of matrix metalloproteinase (MMP)-13 in human chondrocytes[4]. Altered expressions of miR-146a and miR-22 are mediated by inflammatory cytokines[57,156] and widely seen in low-grade osteoarthritis[177], implicating a role in cartilage pathogenesis. MiR-140 has been identified as a direct regulator of aggrecanase-2[106] and has decreased expression in OA chondrocytes. While not yet observed in orthopedic tissues, miR-16 has been shown to directly regulate the expression of a critical inhibitor of apoptosis, B cell lymphoma (BCL)-2[27]. Assessment of the *in vitro* culture media from each cell type for the presence of these miRNAs after cytokine stimulation may allow for the development of non-invasive synovial fluid biomarkers specific to OA progression.

In this study, the time- and concentration-dependent nature of cellular and extracellular miRNAs in chondrocytes, synoviocytes, and meniscus cells as influenced by inflammatory cytokines and the ratio of extracellular miRNA were analyzed as potential indicator of early OA progression.

4.2 Materials and Methods

Cell Culture

Six cadaveric porcine knees from three 35-40kg pigs were obtained from the University of Miami Department of Surgery Tissue Sharing program (Institutional Animal Care and Use Committee approved source) within 2 hours of death. Articular cartilage, meniscus, and synovial membrane tissues were cut into small pieces less than $1mm^3$ and digested with 1mg/mL collagenase (Worthington Biochemical, Lakewood, NJ) and 0.1mg/mL protease (Sigma, St. Louis, MO) at 37°C for 16 hours. Digested cell suspension was strained through a 70µm cell strainer (BD Biosciences, San Jose, CA) and cultured in regular culture media [DMEM (Invitrogen, Grand Island, NY) containing 10% FBS (Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Lonza, Allendale, NJ)] at 37°C in a humidified atmosphere of 5% CO_2. During culture, media was changed every 2-3 days. Cultures of synoviocytes, meniscus cells, and chondrocytes were subjected to experimental procedures at passages 1-3.

Stimulation of Cells with IL-1β and TNF-α

Synoviocytes, meniscus cells, and chondrocytes were plated in triplicate at a density of 10^5 cells per well in a 24-well plate and were allowed to adhere overnight in 1mL regular culture media. Wells of each cell type were washed with 1mL phosphate-buffered saline (PBS) (Lonza, Allendale, NJ) to remove all traces of regular culture media with FBS. To determine the time-dependent cellular and extracellular miRNA expression after cytokine injury, cell types were stimulated for 8, 16, or 24 hours with IL-1β (10ng/mL) or TNF-α (50ng/mL) (both from Peprotech, Rocky Hill, NJ) in 400µL serum-free media [DMEM containing 1% penicillin/streptomycin and 1% ITS-Premix

Supplement (BD Biosciences, San Jose, CA)]. To determine the concentration-dependent cellular and extracellular miRNA expression after cytokine injury, chondrocytes were stimulated for 8 hours with IL-1β (10 or 20ng/mL) or TNF-α (50 or 100ng/mL) in 400μL serum-free media.

The use of serum-free media eliminates the possibility of contamination with exogenous bovine miRNAs from serum[169]. While eliminating serum from cell culture media is a strong biological stimulus on its own, the cell cycle arrest or death commonly associated with serum depletion occurs mostly after 48 hours of culture[52,178]. Normalization to the control group would eliminate any effects of serum depletion from the data. Cells in control group were left unstimulated in 400μL serum-free media. This study utilized 3 groups per cell type per time point: control, IL-1β stimulation, and TNF-α stimulation.

Ratio of extracellular miRNA

We examined the ratio of extracellular miRNAs as a method to determine differential expression without the need for an internal control by Equation 1, where *Ct* is the Real-Time PCR cycle number for the miRNA of interest per sample. Ratios of interest were as follows: miR-146a to miR-140, miR-22 to miR-140, miR-16 to miR-146a, miR-16 to miR-140, miR-22 to miR-16, and miR-22 to miR-27b. Media samples from chondrocytes, meniscus cells, and synoviocytes were taken for this study from the 8 hour time point of the time-dependency study described above. Significant differences between the ratios in the cytokine-stimulated group and the control group were determined by a two-sample Student's t-test.

$$Ratio = \frac{2^{-Ct\ (miRNA\ A)}}{2^{-Ct\ (miRNA\ B)}}$$

Equation 1

Viable Cell Count Analysis

To ensure consistent cell number within time points, 3 cell types were plated in triplicate as described above and stimulated with 10ng/mL IL-1β or 50ng/mL TNF-α for 8, 16, and 24 hours. Chondrocytes were also stimulated with 20ng/mL IL-1β or 100ng/mL TNF-α for 8 hours. Immediately after each time point, the culture media was removed from the wells, and the cells were incubated in PBS containing 1 μmol/L calcein AM from the Live/ Dead Viability/Cytotoxicity Kit (Invitrogen). Staining was visualized on an inverted fluorescent microscope with 495 nm/515 nm excitation/emission for calcein (live cells). Images were taken from the center of well in the 24-well plate through a 10X objective lens. The images were analyzed with ImageJ software, and total number of live cells/image was determined.

RNA Isolation and Quantitative Real-Time PCR

Total RNA from cells was extracted using 250μL per well Tri Reagent per manufacturer's instruction (Molecular Research Center, Cincinnati, OH). To isolate extracellular miRNAs from media samples, the media from each well in each group was combined (1.2mL) and concentrated 10 times using 10kDa MWCO columns (Millipore, Darmstadt, Germany), as described by Turchinovich et al[162]. Extracellular miRNAs from concentrated media samples were isolated using RNeasy Isolation kit (Qiagen, Venlo, Netherlands) per manufacturer's protocol. Prior to first spin in the protocol, 5pg of

synthetic miRNA-39 from *Caenorhabditis elegans* (cel-miR-39) were added as a spike-in control for media samples. RNA was eluted in 30µL of RNase-free water.

For miRNA expression analyses, the qScript microRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) was used to polyadenylate miRNAs and synthesize cDNA from total RNA following manufacturer's instructions. For cell samples, 0.1µg of total RNA containing miRNA was used for reverse transcription. For media samples, a fixed volume of 7µL of total RNA from the 30µL eluate of a given sample was used for reverse transcription. Genetic expression of selected miRNA and mRNA (Table 6) was analyzed by SteponePlus (Applied Biosystems, Grand Island, NY) quantitative real-time PCR system. Expression of RNU6B was used as an endogenous control for miRNA expression in cells. Quantification of expression levels was determined by the $2^{-\Delta\Delta Ct}$ method[90] and normalized to expression levels of respective control group.

Table 6 Quantitative PCR Primers

Gene	Primer Sequences	Reference/ miRbase #
cel-miR-39-3p	5'-TCACCGGGTGTAAATCAGCTTG-3'	MIMAT0020306
hsa-miR-146a-5p	5'-TTTGAGAACTGAATTCCATGGGTTG-3'	MIMAT0000449
hsa-miR-22-5p	5'-AGTTCTTCAGTGGCAAGCTTTA-3'	MIMAT0000077
hsa-miR-16-1-3p	5'-TAGCAGCACGTAAATATTGGCG-3'	MIMAT0004489
hsa-miR-140-5p	5'- AGTGGTTTTACCCTATGGTAG-3'	MIMAT0000431
hsa-miR-27b-5p	5'- AGAGCTTAGCTGATTGGTGAACA -3'	MIMAT0004588
RNU6B	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3' R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'	[167]
18S	F: 5'-CGGCTACCACATCCAAGGA-3' R: 5'-AGCTGGAATTACCGCGGCT-3'	[164]
CASP3	F: 5'-GCTGCAAATCTCAGGGAGAC-3' R: 5'-GGCAGGCCTGAATTATGAAA-3'	[164]

Statistical Analysis

Five independent experiments with different cell preparations (n=5) for each cell type were performed per time point and per stimulation concentration, each with different cell preparation. The statistical significance of the time-dependent differences in miRNA expression per cell type was calculated using two-sample Student's t-test (MATLAB R2014a). The statistical significance of viable cell number and the concentration-dependent differences in miRNA expression was calculated using analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test. All tests were two-tailed and p-values <0.05 were considered statistically significant.

4.3 Results

Time-dependent miRNA expression of knee tissue cells after cytokine stimulation

Several trends were observed in cellular miRNA expressions among cell types elicited by the pro-inflammatory cytokines. MiR-146a was the only miRNA to be significantly up-regulated in all three cell types by both IL-1β and TNF-α at 8, 16, and 24 hours (Table 7 and Table 8 for p-values). In contrast, in all cells stimulated by IL-1β or TNF-α, cellular expression of miR-27b was significantly down-regulated or tended for down-regulation at 8, 16, and 24 hours with the exception of IL-1β-stimulated chondrocytes and meniscus cells at 24 hours. Notably, there was an observed TNF-α-dependency in cellular miR-16 expression with significant down-regulation of miR-16 at multiple time points, while no significant difference in miR-16 expression was found in IL-1β-stimulated cells at any time point. Chondrocyte and meniscus cell expressions of miR-140 were significantly down-regulated at only 24 hours by IL-1β and TNF-α, while

synoviocyte expression of miR-140 was significantly down-regulated at only 8 hours by IL-1β and TNF-α.

Time-dependent release of extracellular miRNA by knee tissue cells after cytokine stimulation

Extracellular miRNA release rate after stimulation differed among the cell types. Notably, chondrocytes had a distinct extracellular miRNA profile after cytokine stimulation as compared to the other two tissues (Table 9 and Table 10 for p-values). While there was a general decline of most extracellular miRNA release rate beyond the 8 hour time point, only extracellular miR-27b increased over time in chondrocyte media after IL-1β and TNF-α stimulation (Figure 4.1A&B). At 8 hours, release of extracellular miR-140 in meniscus cell and synoviocyte media were significantly lower than that in chondrocyte media in control (p=0.009 and 0.025, respectively) and IL-1β-stimulated groups (p=0.009 and 0.05, respectively) (Figure 4.1C). Extracellular miR-140 in TNF-α-stimulated meniscus cell media was significantly lower than that in chondrocyte and synoviocyte media (p=0.008 and 0.032, respectively). Within each time point and cell type, the number of viable cells per well in each group was not significantly different from each other (data not shown).

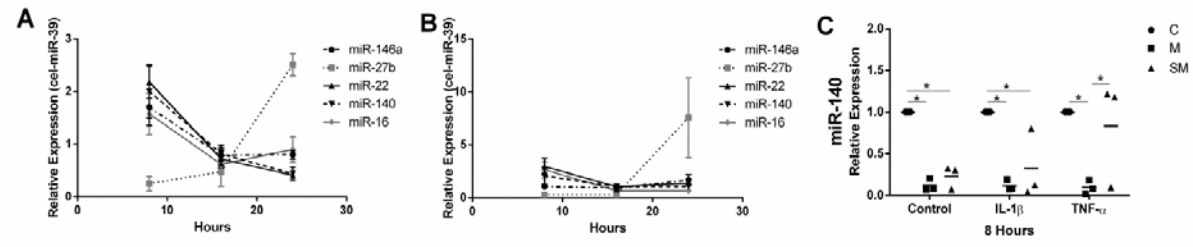

Figure 4.1 - Relative miRNA spectra in chondrocyte culture media after stimulation with (A) IL-1β and (B) TNF-α as compared to control media samples (n=5). (C) Relative expressions of extracellular miR-140 in meniscus cell and synoviocyte media at 8 hours hours as normalized to chondrocyte media (n=3). C, chondrocytes; M, meniscus cells; SM, synoviocytes. * indicates significance p<0.05. Please refer to text for exact p-values. Significance for A and B are shown in Table 4 and 5.

Table 7 Relative expressions of cellular miRNAs in chondrocytes, meniscus cells, and synoviocytes after stimulation with 10ng/mL IL-1β for 8, 16, and 24 hours as compared to control samples at respective time point. P-values < 0.05 were considered significant.

	8 Hours			16 Hours			24 Hours		
Chondrocytes +10ng/mL IL-1β									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	1.09	0.11	0.6158	0.86	0.12	0.2132	0.80	0.17	**0.0378**
miR-16	0.92	0.12	0.2208	0.90	0.09	0.1861	1.24	0.33	0.1844
miR-146a	33.2	10.90	**0.0048**	16.5	4.20	**<0.001**	13.63	4.26	**<0.001**
miR-140	1.07	0.09	0.8109	0.90	0.12	0.2999	0.58	0.19	**0.0012**
miR-27b	0.73	0.10	**0.0028**	0.76	0.09	0.0598	0.96	0.24	0.7279
Meniscus Cells +10ng/mL IL-1β									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	1.27	0.12	**0.0040**	1.19	0.06	0.1140	0.78	0.10	0.1647
miR-16	1.13	0.13	0.2025	1.17	0.17	0.3416	0.89	0.19	0.2164
miR-146a	7.56	2.64	**0.0011**	6.36	1.60	**<0.001**	4.34	1.56	**<0.001**
miR-140	1.14	0.18	0.4299	1.20	0.22	0.3679	0.60	0.23	**0.0484**
miR-27b	0.74	0.08	**<0.001**	0.78	0.08	**0.0081**	1.34	0.51	0.9380
Synoviocytes +10ng/mL IL-1β									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	1.26	0.22	0.0922	1.05	0.12	0.9816	0.86	0.15	0.1810
miR-16	1.09	0.12	0.2762	0.96	0.08	0.8550	1.26	0.25	0.2396
miR-146a	9.49	2.71	**<0.001**	7.70	1.63	**<0.001**	10.47	3.07	**<0.001**
miR-140	1.21	0.12	**0.0409**	1.16	0.15	0.4246	1.39	0.47	0.4317
miR-27b	0.83	0.09	**0.0230**	0.74	0.09	**0.009**	0.73	0.15	**0.0401**

Table 8 Relative expressions of cellular miRNAs in chondrocytes, meniscus cells, and synoviocytes after stimulation with 50ng/mL TNF-α for 8, 16, and 24 hours as compared to control samples at respective time point. P-values < 0.05 were considered significant.

	8 Hours			16 Hours			24 Hours		
Chondrocytes +50ng/mL TNF-α									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	0.97	0.15	0.6234	0.73	0.09	**0.0105**	0.67	0.17	**0.0020**
miR-16	1.12	0.24	0.5183	0.76	0.09	**0.0087**	0.95	0.22	0.6909
miR-146a	5.99	1.05	**<0.001**	6.98	1.98	**<0.001**	7.76	3.36	**<0.001**
miR-140	0.74	0.16	0.0534	0.88	0.17	0.3370	0.59	0.14	**<0.001**
miR-27b	0.53	0.06	**<0.001**	0.43	0.07	**<0.001**	0.67	0.17	**0.0023**
Meniscus Cells +50ng/mL TNF-α									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	1.08	0.20	0.5730	1.05	0.16	0.7284	0.54	0.13	**0.0054**
miR-16	0.92	0.23	0.5591	1.08	0.17	0.7352	0.62	0.20	**0.0130**
miR-146a	2.62	0.67	**0.0015**	6.56	0.98	**<0.001**	5.15	2.98	**0.0267**
miR-140	0.83	0.19	0.1575	1.14	0.21	0.5439	0.63	0.18	**0.0371**
miR-27b	0.53	0.09	**<0.001**	0.62	0.11	**<0.001**	0.37	0.10	**0.0028**
Synoviocytes +50ng/mL TNF-α									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	0.77	0.12	**0.0114**	0.91	0.17	0.4501	0.54	0.10	**<0.001**
miR-16	0.57	0.14	**<0.001**	0.90	0.11	0.2646	0.59	0.11	**0.0016**
miR-146a	2.99	0.80	**0.0017**	8.52	1.86	**<0.001**	4.82	0.83	**<0.001**
miR-140	0.65	0.15	**0.0054**	1.10	0.17	0.7398	0.74	0.15	0.1061
miR-27b	0.42	0.06	**<0.001**	0.73	0.19	0.0819	0.38	0.12	**<0.001**

Table 9 Relative expressions of extracellular miRNAs in chondrocyte, meniscus cell, and synoviocyte culture media after stimulation with 10ng/mL IL-1β for 8, 16, and 24 hours as compared to control media at respective time point. P-values < 0.05 were considered significant.

	8 Hours			16 Hours			24 Hours		
Chondrocyte media +10ng/mL IL-1β									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	2.18	0.30	**0.0017**	0.72	0.17	0.1498	0.41	0.05	**<0.001**
miR-16	1.58	0.40	**0.0210**	0.62	0.15	**0.0460**	0.90	0.24	0.6157
miR-146a	1.70	0.35	**0.0130**	0.79	0.19	0.2857	0.80	0.08	**0.0157**
miR-140	2.01	0.51	**0.0370**	0.82	0.11	0.1692	0.44	0.13	**<0.001**
miR-27b	0.25	0.14	**0.0047**	0.48	0.28	0.3590	2.51	0.21	**0.0066**
Meniscus Cell media +10ng/mL IL-1β									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	2.98	0.85	**0.0330**	0.48	0.11	**0.0024**	1.05	0.18	0.7557
miR-16	4.14	1.31	**0.0294**	1.34	0.34	0.3482	0.86	0.16	0.3025
miR-146a	5.51	3.28	0.1541	0.89	0.21	0.6067	2.12	0.75	0.0929
miR-140	4.75	2.01	0.0691	0.94	0.29	0.8443	0.63	0.17	**0.0284**
miR-27b	0.50	0.15	**0.0428**	0.48	0.06	**0.0030**	0.55	0.18	**0.0135**
Synoviocyte media +10ng/mL IL-1β									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	1.25	0.36	**0.0123**	4.04	1.62	0.0969	0.93	0.19	0.6679
miR-16	1.90	0.13	**0.0205**	1.07	0.35	0.8582	0.75	0.10	**0.0164**
miR-146a	3.05	0.18	**0.0376**	4.14	1.97	0.1487	1.15	0.16	0.3302
miR-140	1.27	0.29	**0.0188**	2.19	0.67	0.1078	1.32	0.40	0.3884
miR-27b	0.56	1.10	0.4110	1.26	0.54	0.5967	2.47	0.94	0.0772

Table 10 Relative expressions of extracellular miRNAs in chondrocyte, meniscus cell, and synoviocyte culture media after stimulation with 50ng/mL TNF-α for 8, 16, and 24 hours as compared to control media at respective time point. P-values < 0.05 were considered significant.

	8 Hours			16 Hours			24 Hours		
Chondrocyte media +50ng/mL TNF-α									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	2.95	0.79	**0.0152**	1.03	0.21	0.8812	1.35	0.24	0.0993
miR-16	2.64	0.77	**0.0268**	0.65	0.16	0.0719	0.66	0.16	**0.0307**
miR-146a	1.05	0.16	0.7764	0.90	0.34	0.2857	1.68	0.50	0.1267
miR-140	2.07	0.84	0.1465	1.03	0.16	0.8904	1.06	0.22	0.7401
miR-27b	0.29	0.09	**0.0014**	0.29	0.17	0.0810	7.57	3.79	0.1987
Meniscus Cell media +50ng/mL TNF-α									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	2.04	0.48	**0.0417**	0.44	0.11	**<0.001**	1.22	0.39	0.5010
miR-16	1.41	0.40	0.2827	0.55	0.13	**0.0100**	1.43	0.31	0.1189
miR-146a	1.30	0.28	0.2453	0.58	0.18	**0.0536**	0.89	0.16	0.4263
miR-140	2.27	0.58	**0.0419**	0.49	0.16	**0.0144**	1.13	0.10	0.1287
miR-27b	1.05	0.33	0.9062	0.61	0.46	0.2157	1.85	0.64	0.1207
Synoviocyte media +50ng/mL TNF-α									
	Mean	**SE**	**P**	**Mean**	**SE**	**P**	**Mean**	**SE**	**P**
miR-22	1.29	0.19	0.1297	2.52	0.87	0.1198	1.55	0.70	0.3319
miR-16	1.35	0.13	**0.0155**	0.85	0.14	0.3796	4.16	2.34	0.1162
miR-146a	1.30	0.21	0.1512	1.34	0.50	0.1491	3.60	1.69	0.2490
miR-140	0.94	0.41	0.8809	1.51	0.50	0.3207	3.60	1.69	0.1178
miR-27b	2.11	0.87	0.2428	1.84	0.87	0.3103	1.50	0.83	0.2532

Ratio of extracellular miRNA in stimulated culture media

At 8 hours after stimulation with 10ng/mL IL-1β and 50ng/mL TNF-α, the ratio of extracellular miR-146a to miR-140 expressions (Figure 4.2A) of chondrocyte media was significantly decreased as compared to control (p=0.025 and 0.036, respectively). However, the ratio of extracellular miR-146a to miR-140 expressions was significantly increased in IL-1β-stimulated synoviocyte media as compared to the control (p=0.045). The ratio of extracellular miR-22 to miR-140 expressions was significantly increased only in chondrocytes stimulated with TNF-α (p=0.025) (Figure 4.2B). The ratio of extracellular miR-16 to miR-146a expressions was significantly increased in TNF-α-stimulated chondrocyte media (p= 0.003) whereas significantly decreased in IL-1β- and TNF-α-stimulated synoviocyte media (p=0.017 and 0.019, respectively) (Figure 4.2C). The ratio of extracellular miR-22 to miR-27b expressions was significantly increased only in IL-1β-stimulated chondrocyte media (p=0.010) (Figure 4.2D). However, no significant differences were found in any extracellular miRNA ratio for IL-1β- and TNF-α-stimulated meniscus cell medium after 8 hours.

At 16 hours, the ratio of extracellular miR-22 to miR-140 expressions (Figure 4.3A) was significantly decreased in IL-1β- and TNF-α-stimulated chondrocyte media as compared to control (p=0.025 and 0.032, respectively). The ratio of extracellular miR-16 to miR-146a expressions (Figure 4.3B) was also significantly decreased in IL-1β- and TNF-α-stimulated chondrocyte media at 16 hours as compared to control (p=0.015 and 0.004, respectively). After 16 hours of stimulation with IL-1β, the ratio of extracellular miR-22 to miR-16 expressions was significantly decreased (p=0.043) (Figure 4.3C).

However, no significant differences were found in any extracellular miRNA ratio for IL-1β- and TNF-α-stimulated synoviocyte and meniscus cell medium after 16 hours.

At 24 hours, the ratio of extracellular miR-16 to miR-140 expressions (Figure 4.4A) was significantly increased in IL-1β-stimulated chondrocyte media as compared to control ($p=0.024$). The ratio of extracellular miR-22 to miR-27b expressions (Figure 4.4B) was significantly decreased in TNF-α-stimulated chondrocyte and synoviocyte media at 24 hours as compared to respective controls ($p=0.006$ and 0.002, respectively). After 24 hours of stimulation with TNF-α, the ratio of extracellular miR-22 to miR-16 was significantly decreased ($p=0.043$) (Figure 4.4C). However, no significant differences were found in any miRNA expression ratio for IL-1β- and TNF-α-stimulated meniscus cell medium after 24 hours.

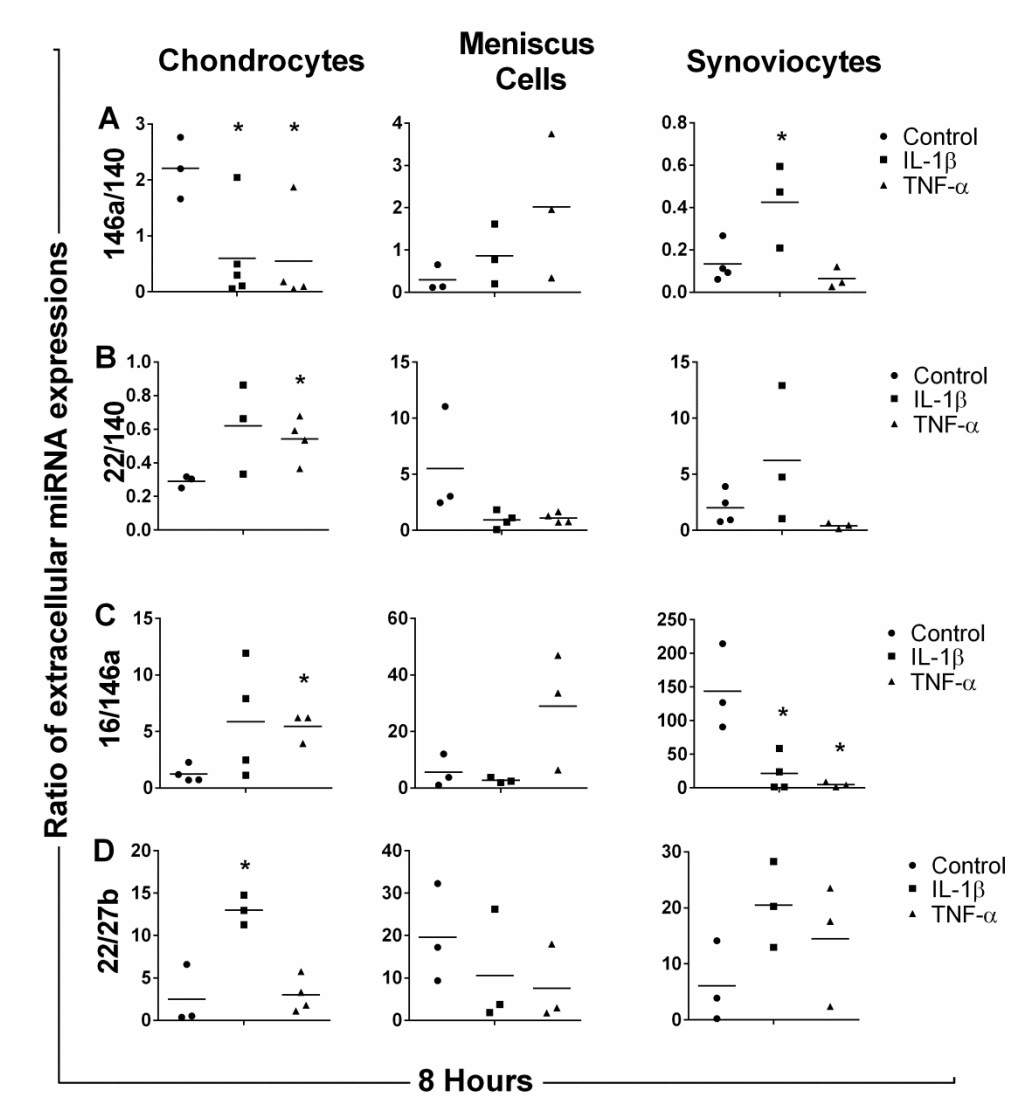

Figure 4.2 - Ratios of extracellular (A) miR-146a to miR-140, (B) miR-22 to miR-140, (C) miR-16 to miR-146a, and (D) miR-22 to miR-27b at 8 hours in control and stimulated (10ng/mL IL-1β or 50ng/mL TNF-α) chondrocyte, meniscus cell, and synoviocyte media. *indicates significant $p<0.05$ as compared to control group (n=3-5). Please refer to text for exact p-values.

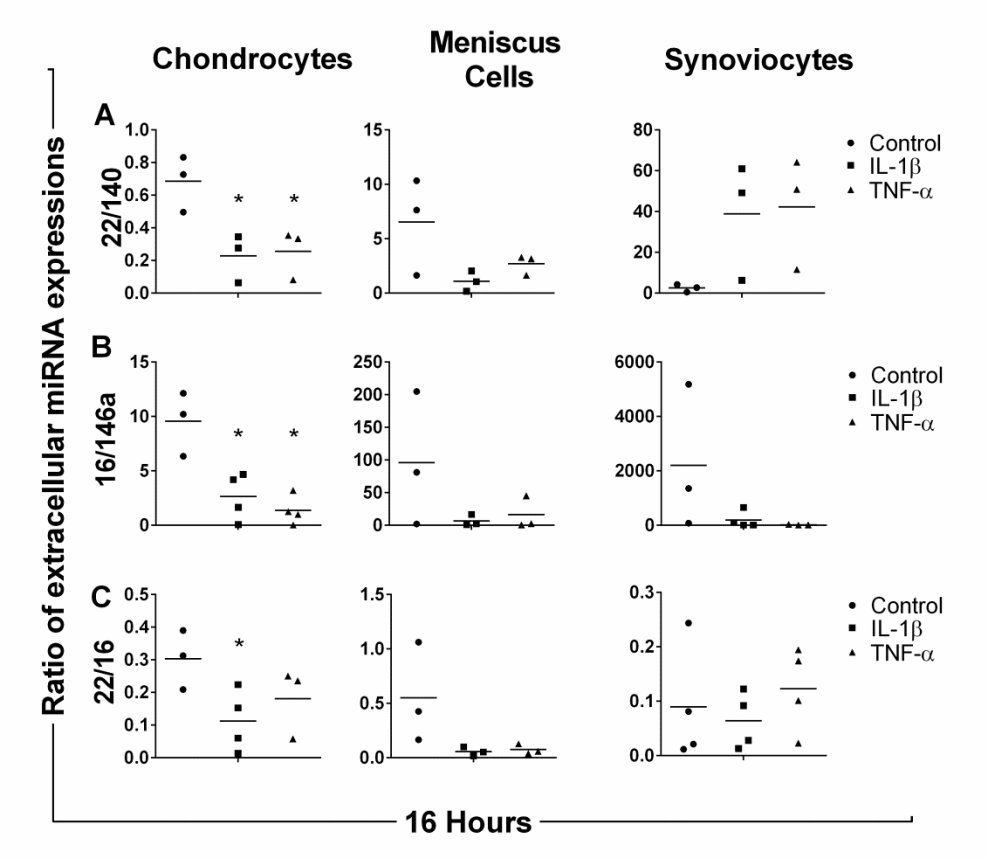

Figure 4.3 - Ratios of extracellular (A) miR-22 to miR-140, (B) miR-16 to miR-146a, and (C) miR-22 to miR-16 at 16 hours in control and stimulated (10ng/mL IL-1β or 50ng/mL TNF-α) chondrocyte, meniscus cell, and synoviocyte media. *indicates significant $p<0.05$ as compared to control group (n=3-5). Please refer to text for exact p-values.

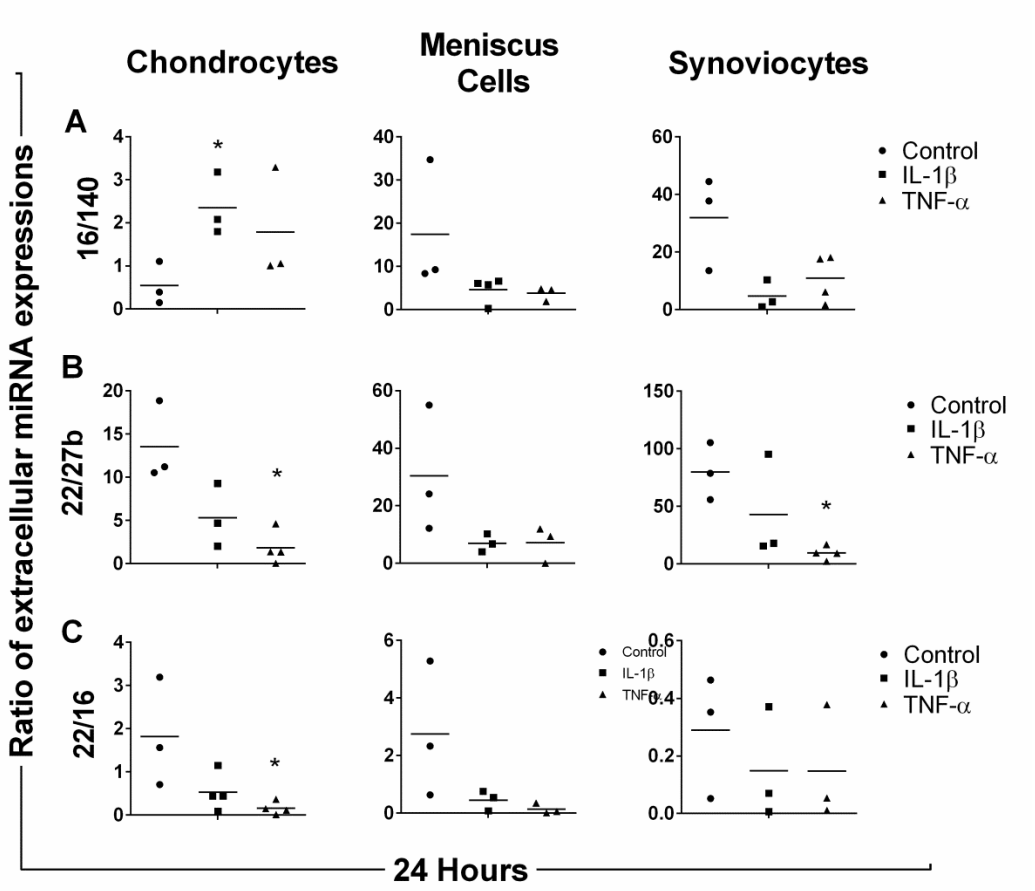

Figure 4.4 - Ratios of extracellular (A) miR-16 to miR-140, (B) miR-22 to miR-27b, and (C) miR-22 to miR-16 at 24 hours in control and stimulated (10ng/mL IL-1β or 50ng/mL TNF-α) chondrocyte, meniscus cell, and synoviocyte media. *indicates significant $p<0.05$ as compared to control group (n=3-5). Please refer to text for exact p-values.

Concentration-dependent miRNA Expression in Chondrocytes after Cytokine Stimulation

Chondrocytes were stimulated with 10 and 20ng/mL IL-1β (Figure 4.5A) and 50 and 100ng/mL TNF-α (Figure 4.5B) for 8 hours. The higher concentration (20ng/mL) of IL-1β significantly up-regulated cellular miR-22, -16, and -140 as compared to the lower IL-1β concentration (10ng/mL) and controls (ANOVA $p=0.003$, <0.001, and <0.001, respectively), whereas the IL-1β of 10ng/mL did not exhibit any significant effects on those miRNA expression. Significant up-regulation of cellular miR-146a was seen at both IL-1β concentrations as compared to control (ANOVA $p<0.001$). Cellular expression of

miR-27b was not significantly affected by the higher concentration of IL-1β, whereas it was significantly down-regulated by the lower concentration of IL-1β as compared to the control (ANOVA $p=0.002$). The same concentration-dependent effects on cellular expressions of miR-22, miR-146a, miR-140, and miR-27b were seen in TNF-α treated groups (all ANOVA $p<0.005$) (Figure 4.5B).

For extracellular miRNA expression, the lower IL-1β concentration significantly up-regulated extracellular miR-22, miR-16 and -146a expressions, whereas the higher IL-1β concentration did not cause significant differential expressions of those miRNAs as compared to control (ANOVA $p<0.001$, 0.03, and 0.002, respectively) (Figure 4.5C). Extracellular miR-27b expression was significantly increased at the higher IL-1β concentration, and however, significantly decreased at the lower IL-1β concentration as compared to control (ANOVA $p=0.002$). Extracellular expression of miR-140 was significantly increased by both IL-1β concentrations as compared to the control (ANOVA $p<0.001$). Stimulation with the higher TNF-α concentration (100ng/mL) significantly up-regulated extracellular miR-16 and miR-146a (ANOVA $p=0.007$ and 0.009, respectively) and exhibited no significant effect on extracellular miR-22 and miR-27b expressions as compared to the control (Figure 4.5D). In contrast, the lower TNF-α concentration (50 ng/mL) significantly up-regulated extracellular miR-16 and miR-22 expressions (ANOVA $p=0.008$ and <0.001, respectively) and exhibited no effect on extracellular miR-146a expression as compared to the control. Extracellular expression of miR-140 was not significantly affected by both TNF-α concentrations.

Concentration-dependent apoptotic expression in chondrocytes after cytokine stimulation

To determine if the change in chondrocyte cellular and extracellular expressions of miRNAs at the different concentrations were due to apoptotic activity, we examined the relative expression of Caspase-3 (CASP3) of stimulated chondrocytes at the various concentrations at 8 hours. After 8 hours of stimulation with 20ng/mL IL-1β, chondrocyte expression of CASP3 was significantly up-regulated as compared to control (ANOVA $p=0.032$, Figure 4.5E). Stimulation of chondrocytes with 100ng/mL TNF-α significantly up-regulated CASP3 expression as compared to control and 50ng/mL TNF-α (ANOVA $p=0.014$) (Figure 4.5F).

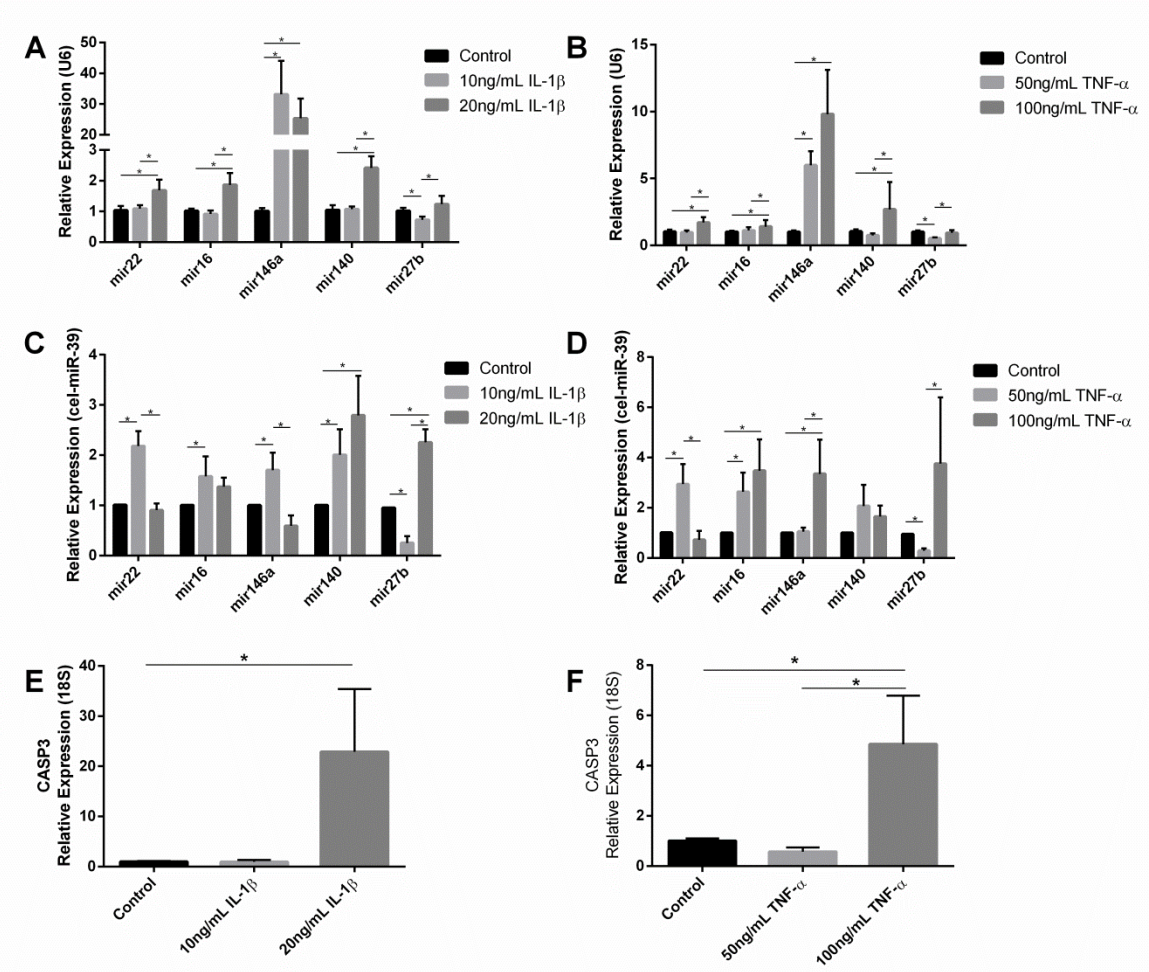

Figure 4.5 – Cellular and extracellular miRNA spectra in chondrocytes after stimulation with 10 and 20ng/mL IL-1β and 50 and 100ng/mL TNF-α at 8 hours. Cellular miRNA spectra in chondrocytes after stimulation with (A) 10 and 20ng/mL IL-1β and (B) 50 and 100ng/mL TNF-α at 8 hours. Extracellular miRNA spectra in chondrocyte culture medium after stimulation with (C) 10 and 20ng/mL IL-1β and (D) 50 and 100ng/mL TNF-α at 8 hours. Relative CASP3 expression in chondrocytes after stimulation with (E) 10 and 20ng/mL IL-1β and (F) 50 and 100ng/mL TNF-α at 8 hours. Data are given as mean±SE. * indicates significance $p<0.05$ (n=5). Please refer to text for exact p-values.

4.3 Discussion

The present study is the first to examine the distinct cellular and extracellular miRNA spectra of chondrocytes, meniscus cells, and synoviocytes after stimulation with inflammatory cytokines. After traumatic joint injury and during OA development, inflammatory cytokine levels, including IL-1β and TNF-α, increase in the joint tissues, inciting cellular apoptosis, up-regulation of catabolic enzymes, and eventual cartilage

degeneration[9,41,43,60,80]. Better understanding of how the presence of inflammatory cytokines influences the genetic regulation and release of miRNAs in the joint tissues may help elucidate the role of miRNAs in OA development.

Prior to release from the cell, extracellular miRNAs are bound to protective proteins or encapsulated in exosomes which increase their stability in nuclease-rich environments, such as serum, synovial fluid, and culture media[73,169,176]. Ceramide, a bioactive sphingolipid that is regulated by neutral sphingomyelinase 2, has been shown to be responsible for packing miRNA into exosomes in the cytosol for transport[71]. Nucleophosmin 1 (NPM1) is an RNA-binding protein that binds to miRNA, protecting it from RNase degradation, and has been shown to be released with miRNA into the extracellular space[115,169]. Previous studies suggest that extracellular release of individual mRNAs is directly proportional to their intracellular expressions[42,71,171]. This mechanism was observed in several miRNAs investigated in this study, such as miR-27b in IL-1β- and TNF-α-stimulated chondrocytes at 8 hours, and miR-22 and -140 in IL-1β-stimulated chondrocytes at 24 hours. However, some extracellular miRNA expression examined (e.g., miR-146a and miR-16) did not follow the mechanism described above. As suggested by previous studies[73,74,121,126,169,183], cells may "selectively" export miRNA. For example, in chondrocytes stimulated with 10ng/mL IL-1β for 8 hours, we observed significant miR-146a up-regulation inside and outside the cell. However, while up-regulation of cellular miR-146a expression was still seen, extracellular expression of miR-146a was down-regulated in chondrocyte media as compared to the control after 24 hour IL-1β treatment. It has been shown that expressions of the target mRNAs of miR-146a, such as IRAK1, were up-regulated by IL-1β [168]. As time progresses and the up-

regulation of its target mRNAs under IL-1β treatment, miR-146a becomes more "active" in the post-transcriptional process in attempt to inhibit the target mRNAs, which is reflected in the decreased release rate at 24 hours. Another example involves miR-16, which directly regulates BCL-2, a potent apoptosis inhibitor[27]. In chondrocytes stimulated with 10ng/mL IL-1β for 8 hours, there was no significant increase in apoptotic CASP3 expression, but there was a significantly increased release rate of miR-16 (Figure 4.5 C and E), indicating relative "inactivity" of its inhibition of BCL-2. However, under apoptotic conditions where CASP3 was significantly up-regulated after 8 hours of 20ng/mL IL-1β stimulation, miR-16 expression is up-regulated in the chondrocytes as its release rate decreases, indicating increased "activity" of its inhibition of BCL-2 (Figure 4.5 C and E). Based on these results, the selective release process may occur when a specific miRNA is relatively inactive in the post-transcription regulation than the other miRNAs present in the cytosol. Therefore, the extracellular miRNA spectra of stimulated or diseased cell may reflect the post-transcriptional activity of a specific miRNA[71,119,171].

Extracellular miRNA expression analysis requires a suitable endogenous reference miRNA to minimize variation caused by different experimental conditions or sample source by normalization[107]. This task has proven difficult over the years with many reports claiming multiple suitable reference miRNAs specific for each tissue[46,107,170,175]. Currently, many extracellular miRNAs are used as references to normalize miRNAs in bodily fluid samples[46,170,175]. In this study, we observed multiple ratios of extracellular miRNAs that were significantly altered by the presence of IL-1β and TNF-α. By utilizing the ratio of miRNAs in a sample, the need for an endogenous reference miRNA is eliminated. Also it was found that the cell types examined in our

study exhibited different patterns of extracellular miRNA ratios (Figure 4.2, Figure 4.3, Figure 4.4). The results suggest a method to examine the cellular status of chondrocytes for the early detection of OA or cartilage injury by analyzing extracellular miRNA in synovial fluid. Since only 5 miRNAs were examined in this study, more ratios from a larger miRNA pool will be investigated in future studies.

Although the miRNAs investigated in this study had been previously shown to be differentially expressed in IL-1β-stimulated or OA chondrocytes[105], this study also examined the intra- and extra-cellular expressions of the miRNAs of synoviocytes and meniscus cells and compared three cell types. Multiple studies reported a significant increase in miR-146a expression in IL-1β-stimulated and OA chondrocytes and synoviocytes[81,168,177], which was observed in our study not only in TNF-α and IL-1β-stimulated chondrocytes and synoviocytes, but also in TNF-α and IL-1β-stimulated meniscus cells. MiR-140, regulator of aggrecanase-2[106], was significantly decreased in both TNF-α and IL-1β-stimulated chondrocytes and meniscus cells, but not in stimulated synoviocytes, which can be attributed to the fact that meniscus and cartilage tissues naturally contain more aggrecan than synoviocytes[172]. Expression of miR-27b, direct regulator of MMP-13[4], was significantly down-regulated in all cell types by both TNF-α and IL-1β. While synovial tissue is not comprised of collagen II as are cartilage and meniscus tissues, it is possible that the down-regulation of miR-27b in synoviocytes indicates an increased production of MMP-13 to be released into the synovial fluid[127]. Because the joint capsule is an enclosed environment, the altered genetic and miRNA expressions in tissues surrounding cartilage can potentially affect and propagate an acute injury or early OA development via paracrine action[40,71,73]. Extracellular miRNA

packaged in exosomes have also been shown to be readily taken up by surrounding cells[40]. Therefore, it is necessary to examine both the intra- and extracellular miRNA profiles of all joint tissues under inflammatory or diseased conditions.

A limitation of this study is the small number of OA-associated miRNA expressions examined in cells and culture media. Therefore, future studies should further investigate the cellular and extracellular miRNA spectras from the three joint tissues using microarray technology. We also acknowledge that the concentrations of inflammatory cytokines used to stimulate the cells may not be representative of physiological injury or OA conditions. Additionally, we did not address the mRNA expressions which are known to be regulated by the selected miRNAs, since the expressions of the target mRNAs have been studied previously under similar treatment conditions[4,27,57,106,177] and this study primarily focused on the cellular and extracellular miRNA expressions after cytokine stimulation. In spite of these limitations, we believe that the present study has provided valuable information concerning the basic science of miRNA expression in joint tissues.

In summary, our results demonstrated chondrocytes, meniscus cells, and synoviocytes have distinct extracellular miRNA spectra and patterns of extracellular miRNA ratios under inflammatory conditions. Thus, analysis of the ratio of the extracellular miRNAs could be used as a potential method of evaluating genetic changes within specific cells. As miRNAs are exported into the extracellular environment in response to injury or disease conditions, characterization of their expression in the media and synovial fluid could lead to the development of better early detection strategies of OA or cartilage injury.

Chapter 5
Ratio of Synovial Fluid microRNA as Method of Early Detection of Acute Cartilage Injury: Proof of Concept

5.1 Background

Post-traumatic osteoarthritis (PTOA) accounts for 12% of OA cases nationally and is usually diagnosed after the cartilage has begun to degenerate and cause disabling pain to the patient[22]. A number of studies have suggested biomarkers of cartilage injury by examining the expression of molecular by-products of cartilage degeneration, such as cytokines and matrix fragments[24,104,152]. Other methods currently under investigation of efficacy include quantification of biochemical markers in the synovial fluid, such as nitrotyrosine, tenascin-C, CD-14, OPN, S100A8/A9 proteins, a range of interleukins, and others[25,47,61,63,89,104,113]. While some of the listed biomarkers showed promise in successfully discriminating between degenerated and healthy knees, they all required the cartilage to have some level of progressive degeneration and most importantly, the tissue source of the biomarkers were unknown. Thus, there is a substantial need for a biomarker for early detection of cartilage injury prior to the onset of cartilage degeneration for the prevention of PTOA.

Several studies have described the use of extracellular microRNAs (miRNAs) in the blood or serum as biomarkers of various cancers and chronic diseases[159,177] (Review; Brase et al.[20]). Additionally, a novel study by Murata et al.[110] reported that miRNAs were exported into the synovial fluid in a stable form and that osteoarthritic synovial tissues released miRNAs into culture media at similar concentrations to what is found in osteoarthritic synovial fluid. Therefore, differential expressions of miRNAs in the

synovial fluid may be suitable as potential non-invasive biomarkers of acute cartilage injury.

As shown in Chapter 4, chondrocytes, meniscus cells, and synoviocytes release miRNA in different patterns when stimulated with inflammatory cytokines IL-1β and TNF-α. We also identified a method of examining differential miRNA expression in the culture media by comparing the ratios of extracellular miRNAs, thereby eliminating the need for an extracellular reference gene in fluid samples. In this proof of concept study, the ratios of synovial fluid miRNAs at 8 hours after acute cartilage injury were examined using the ex-vivo intact joint impact model.

5.2 Materials and Methods

Acute cartilage injury

The ex-vivo intact joint injury model described previously was used in this study (Chapter 2.2). One hour after impact, 1 mL of Phosphate Buffered Saline (PBS) was administered to injured and control knees via intra-articular injection. At 8 hours after injury, synovial fluid was harvested and stored at -80°C for analyses of miRNA expressions.

Analysis of miRNA expressions in synovial fluid

Total RNA from about 200μL of synovial fluid was extracted using 1mL Tri Reagent per manufacturer's instruction (Molecular Research Center, Cincinnati, OH). The same volume of synovial fluid from control and injured knees was used for RNA extraction. Prior to the addition of chloroform in the protocol, 5pg of synthetic miRNA-39 from *Caenorhabditis elegans* (cel-miR-39) were added as a spike-in control. The qScript microRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) was

used to polyadenylate miRNAs and synthesize cDNA from total RNA following manufacturer's instructions. A fixed volume of 7μL of total RNA of a given sample was used for reverse transcription. Expression of miRNA (Table 6) was analyzed by SteponePlus (Applied Biosystems, Grand Island, NY) quantitative real-time PCR system. Quantification of expression levels was determined by the $2^{-\Delta\Delta Ct}$ method[90] and normalized to expression levels of cel-miR-39 to account for sample processing variation.

Ratio of synovial fluid miRNA

Based on the method previously described in Chapter 4, we examined the ratio of extracellular miRNAs by Equation 1. Because different ratios were significant at different time points (Figure 4.2-4), the combination of two ratios was determined by Equation 2, where *Ct* is the Real-Time PCR cycle number for the miRNA of interest per sample. All possible individual ratios and combinations of two ratios were analyzed.

$$\boldsymbol{Ratio = \frac{2^{-Ct\,(miRNA\,A+miRNA\,C)}}{2^{-Ct\,(miRNA\,B+miRNA\,D)}}}$$

Equation 2

Statistical Analysis

Comparison of synovial fluid miRNA expressions from control (n=4) and injury (n=4) groups was performed by a two-sample Student's t-test (GraphPad Prism 6). p-value <0.05 was considered significant.

5.3 Results and Discussion

At 8 hours after impact injury, miRNA expressions in injured synovial fluid were not significantly different from that of control (Figure 5.1). At 8 hours after impact injury, the ratio of miR-146a to miR-140 in injured synovial fluid was significantly lower

than control (p=0.029) (Figure 5.2). At 8 hours after impact injury, four combinations of miRNA ratios in injured synovial fluid were significantly lower than control (Figure 5.3).

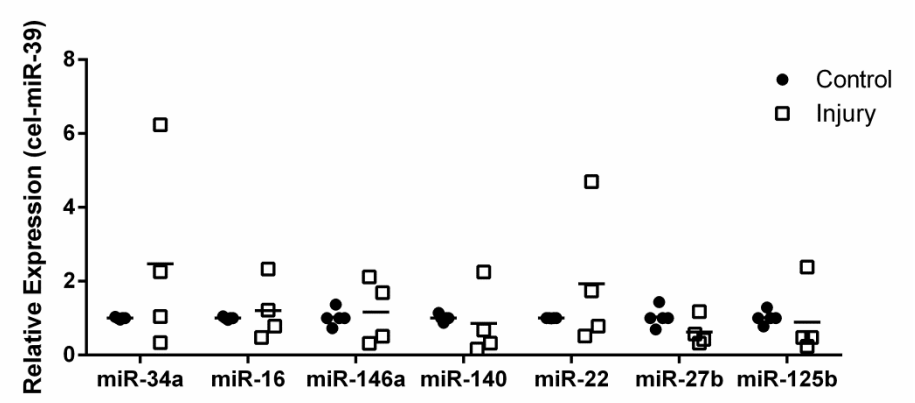

Figure 5.1 - Relative expressions of miRNAs in synovial fluid at 8 hours after impact injury.

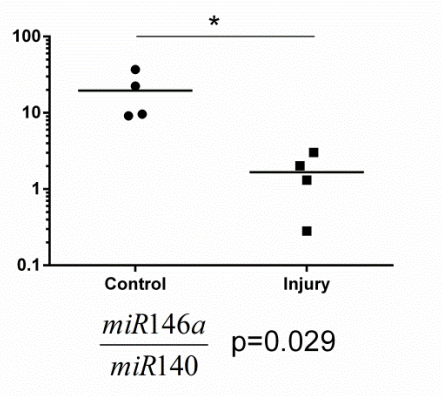

Figure 5.2 - Ratio of synovial fluid miRNAs at 8 hours after impact injury. *indicates significant p<0.05 as compared to control group (n=4-5).

In this proof of concept study, the findings indicate that the ratio of synovial fluid miRNAs could be used as a method of early detection of acute cartilage injury. Consistent with the in-vitro cellular experiment (Chapter 4), the ratio of miR-146a to miR-140 in injured synovial fluid was significantly lower than in uninjured synovial fluid. Only in chondrocytes after simulated injury at 8 hours was this ratio decreased (Figure 4.2). Therefore, it reasonable to suggest that decrease in miR-146a to miR-140

expression may be an indicator of cartilage injury. Moreover, when the ratio of miR-146a to miR-140 is multiplied to ratios of other miRNAs, the new ratio can be considered as normalized to cartilage.

When analyzing the relative expressions of miRNAs, a spike-in miRNA control (cel-miR-39) was used to account for variation in the sample processing. However, it does not account for variation in individual pig joints, such as total synovial fluid volume, injury conditions, or initial health of joint, which may explain the lack of significant findings in our expression data. Through the comparison of same sample miRNA ratios, the need for a reference gene is eliminated.

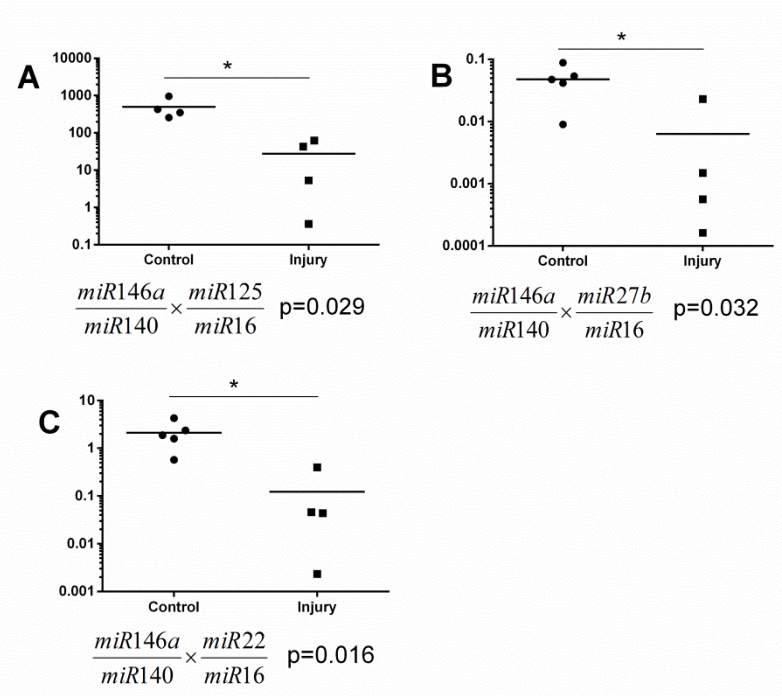

Figure 5.3 – Combination of ratios of synovial fluid miRNAs at 8 hours after impact injury. *indicates significant p<0.05 as compared to control group (n=4-5).

There are several future directions for this study. First, we examined the miRNA expressions in synovial fluid at only one time point due to the nature of the ex-vivo intact joint impact injury model. A long term in-vivo impact injury study is necessary to

determine how active synovial fluid replenishment affects the ratios of miRNA expressions at and beyond 8 hours. Second, since only a small number of miRNAs were investigated, microarray technology can be used to discover more differentially expressed miRNA ratios in synovial fluid. In general, the present proof-of-concept study with this injury model has provided a valuable starting point for further research into the use of synovial fluid miRNAs as biomarkers of acute cartilage injury.

In summary, the results proved the concept that the ratios of miRNAs in the synovial fluid were differentially expressed at 8 hours after acute cartilage injury. With the support of the previous study (Chapter 4), the ratio of the synovial fluid miRNAs could be used as a potential method of early detection of cartilage or joint injury. The next logical step in this continuum of research is to conduct long-term in-vivo impact injury studies to evaluate the differential expressions of synovial fluid miRNAs. As synovial fluid miRNAs are better characterized, potential miRNA biomarkers and their role in OA progression may be elucidated.

Chapter 6
Conclusion and Future Directions

6.1 Recommendations for future work

While this research has demonstrated potential methods of early detection and intervention for acute cartilage injury, many opportunities for extending the scope of this work remain. This section presents some of these directions.

Microarray technology for the discovery of differentially expressed miRNA

In Chapter 2, we selected a panel of 7 miRNA from the literature that have been shown to play a role in the pathogenesis of PTOA and/or its symptoms to be evaluated in the successive studies. However, as shown in Chapter 1, there are many miRNAs implicated in the development of PTOA and potentially many more remain unknown. The use of microarray technology with a panel of over 3000 miRNAs would allow for the discovery of miRNA differentially expressed in knee joint tissues and synovial fluid after acute cartilage impact injury. Furthermore, the microarray analysis may reveal new miRNA ratios in the synovial fluid that may contribute to the discovery of a biomarker of acute cartilage injury.

In-vivo porcine acute cartilage injury model

As mentioned in Chapter 3, the ex-vivo intact joint injury model is limited by the viability of the tissues without blood supply. Due to this limitation, we are unable to evaluate the progression of the cartilage injury into OA, as well as evaluate the long-term efficacy of the treatments administered after injury. Therefore, the next logical step in this body of research is to confirm and expand upon the results from the ex-vivo studies in longitudinal porcine *in-vivo* studies. With an in-vivo study, varying doses of treatments will be needed to determine optimal doses for long-term effect *in-vivo*. Samples of

synovial fluid can be taken throughout the duration of the study to determine the prognosis of the treatments by examining differential synovial fluid miRNA expressions over time.

Examine miRNA ratios in human synovial fluid

In Chapter 4 and 5, we introduced the method of using ratios of extracellular miRNAs as indicators of cellular injury. Future studies implementing this method may include examining the ratios of miRNAs from synovial fluid from healthy, injured, and osteoarthritic human joints. This study may confirm the findings from the porcine ex-vivo and in-vivo studies as well as elucidate potential biomarkers of joint injury and early OA. An anticipated problem could be that in human joint injuries, the cartilage may not be the only tissue injured, but the injury may involve ligament and/or meniscus tears which may introduce blood into the synovial fluid which may mask extracellular miRNA released by the injured tissues. Therefore, new reliable analysis needs to be developed to distinguish different origins of extracellular miRNA.

Anti-oligonucleotides and miRNA mimics as early intervention strategies

In Chapter 3, we proposed a miRNA mechanism of IRAP treatment after acute cartilage impact injury. Intra-articular injection of anti-oligonucleotides and/or miRNA mimics in the ex-vivo injury model will further elucidate the miRNA mechanism of IRAP treatment.

6.2 Dissertation Conclusion

As stated in the introduction, the goal of this dissertation research was to investigate potential early detection and intervention strategies for acute cartilage injury based on the miRNA profiles in knee joint tissues and synovial fluid. In Chapter 2, the

ex-vivo porcine intact joint impact injury model was developed to injure the cartilage and meniscus simultaneously in a physiologically compressive manner while maintaining the joint capsule intact. The study also demonstrated that impact injury causes aberrant regulation of degenerative enzymes and inflammatory cytokines in cartilage, along with a decrease in chondrocyte viability, which may increase the risk of PTOA development. In Chapter 3, the findings demonstrated that early intervention treatments with IRAP and HA administered during acute phase of cartilage impact injury modulates the catabolic microRNA and gene expression in cartilage and meniscus, while DEX may only modulate gene expression. In Chapter 4, the results demonstrated chondrocytes, meniscus cells, and synoviocytes have distinct extracellular miRNA spectra and patterns of extracellular miRNA ratios under inflammatory conditions. Also, the analysis of the ratio of the extracellular miRNAs could be used as a potential method of evaluating genetic changes within specific cells. In Chapter 5, it was determined that the ratios of miRNAs in the synovial fluid were differentially expressed at 8 hours after acute cartilage injury. As synovial fluid miRNAs are better characterized, potential miRNA biomarkers and their association with OA progression may be elucidated.

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