A POTENTIAL BIOLOGIC ROLE FOR THE MENISCUS
IN THE DEVELOPMENT OF OSTEOARTHRITIS.

BY

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Dedication

To my wife, Rebecca, whose enduring patience and unwavering love have made this project possible;

And to my parents, Alan and Kandy, and my brother, Evan, who have always believed in me and offered their perpetual support in my professional pursuits.
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Abstract

The overarching goal of this research is to identify the biologic role of the meniscus in the development of osteoarthritis. We hypothesized that inflammatory factors associated with joint injury would stimulate menisci to increase production of matrix-degrading enzymes, cytokines and chemokines, which could contribute to joint tissue destruction and subsequent development of osteoarthritis. We examined meniscus pathology in established osteoarthritis, characterized the alterations in meniscus cell processes between normal and osteoarthritic menisci, and explored potential pathways involved in osteoarthritis pathogenesis including a novel pathway proposed to mediate osteoarthritis development.

Vervet monkeys were used to examine meniscus pathology as part of the natural history of osteoarthritis and demonstrated osteoarthritic changes. Aged and degenerative menisci secreted increased matrix-metalloproteinases (MMPs) and cytokines and chemokines. To explore osteoarthritis pathogenesis in the human meniscus, meniscus cells were collected from normal tissue donors and patients undergoing total knee arthroplasty. Meniscus cells responded to pro-inflammatory stimulation with increased production of catabolic factors, including MMPs and cytokines and chemokines. Pro-inflammatory stimulation acted at least in part through the nuclear factor-κB (NF-κB) pathway. The hypoxia-inducible factor (HIF) family was then evaluated to determine the HIF family contribution to osteoarthritis pathogenesis in human meniscus cells and evaluate them in the context of a mouse surgical model of osteoarthritis. Human meniscus cells did express HIF family genes, but we could not substantiate a role for the HIF family as a primary regulator of osteoarthritic changes in the meniscus. Heterozygous deletion of the HIF-2α gene, Epas1, in mice did not provide resistance to surgically induced osteoarthritis.
The meniscus likely has a biologic role in the development of osteoarthritis. Pro-inflammatory stimulation and catabolic alterations in the meniscus produce a disease process that appears to be complementary to that of articular chondrocytes. The role of the HIF pathway in promoting osteoarthritic changes in the meniscus is not clear and requires further investigation. Continued exploration into the biologic responses of the meniscus to injury and may yield improved repairs and provide opportunities for tissue engineering and may ultimately aid in prevention or attenuation of osteoarthritis.
Introduction to Osteoarthritis and the Meniscus

Austin V. Stone, MD


**Introduction**

**Scope of the Problem**

Osteoarthritis is a tremendous health burden affecting more than 27 million Americans at an annual economic cost exceeding $128 billion.\(^1\) Osteoarthritis risk increases with age, obesity, joint injury, and participation in intensive physical training\(^2\)-\(^5\). Osteoarthritis was estimated to affect more than 27 million people in 2008 and the burden of osteoarthritis continues rise with expectation that more than 67 million people (25% of adults) will be affected by year 2030\(^2\)-\(^6\).

The knee is a common and debilitating site for osteoarthritis. In an analysis of population based studies, osteoarthritis of the knee was found to affect between 14.8% and 37.4% of adults depending on age\(^6\). Osteoarthritis in the knee results from a catabolic process that leads to a compromise of the structural integrity of the cartilage, subchondral bone, and the meniscus\(^7\)-\(^8\).

The reactive changes and resultant tissue destruction are painful and progressive and frequently require surgical intervention for debilitating symptoms.

**Orthopaedic Surgery and the Meniscus**

Arthroscopic surgery is the most common surgical intervention for meniscus injury performed by orthopaedic surgeons today\(^9\). Meniscus tears may be traumatic or degenerative. Traumatic tears occur more frequently in younger individuals when the meniscus is caught under excessive force, while degenerative changes are usually less symptomatic and occur in older individuals\(^9\),\(^10\). Treatments for meniscus injury include meniscal repair or partial to complete meniscectomy since many tears are irreparable. Total meniscectomy is associated with poor outcomes and increases the relative risk of advanced radiographic knee osteoarthritis development by 14.0 (CI: 3.5 – 121.2) and the procedure is consequently avoided\(^9\),\(^11\),\(^12\). Repair may be attempted and yields improved long-term results for the return to sports activity and a decreased incidence of osteoarthritis\(^13\),\(^14\); however, many tears are not amenable to repair due to poor vascularization of the tissue\(^15\),\(^16\). Although partial meniscectomy produces slightly more
favorable outcomes following meniscus injury, osteoarthritis still risk increases through both a change in knee biomechanics and the initial injury to the meniscus. The relative risk for osteoarthritis development after partial meniscectomy depends on the type of tear, 7.0 (CI: 2.1-23.5) for degenerative tears and 2.7 (CI: 0.9-7.7) for a traumatic tears, when compared to age matched controls. Even patients who decline surgical intervention following meniscal injury have an odds ratio of 5.7 (CI: 3.4-9.4) times more likely to development of radiographic osteoarthritis. An improved understanding of meniscus basic biology and pathologic processes would lead to alternative strategies to managing meniscus injury and improving patient outcomes.

The basic biology of the meniscus.

The knee joint is a diarthrodial joint composed of the femur and tibia and is stabilized by the collateral ligaments, the cruciate ligaments, and medial and lateral meniscus. The meniscus is a fibrocartilaginous structure that aids in load distribution and stabilization of the knee. Each meniscus is crescent shaped and anchored to the tibial plateau by the ligamentous anterior and posterior horn with additional femoral stabilization via the meniscus femoral ligaments. The meniscus is wedge-shaped in radial cross-section between the anterior and posterior horn. The inferior portion of the meniscus lies flat against the surface of the tibia, while the superior portion accommodates the contour of the femoral condyles. The shape of the meniscus allows it to act as a stabilizer in the cam function of the femur providing load sharing through distribution of joint contact stresses and limiting extremes in knee joint motion. Additionally, the meniscus has a complex neurovascular structure: the outer one-third of the meniscus is highly vascular and innervated, while the inner two-thirds are avascular. This neurovascular pattern greatly compromises the tissue’s ability to heal.

A unique and highly organized structure enables the meniscus to provide exceptional load distribution and enhance stability. Compressive forces are absorbed by the high water content (approximately 70%) of the meniscus and its collagen structure. The collagen content in the
meniscus is reported to be between 60% and 75% of the dry weight of the meniscus. While articular cartilage is comprised of type II collagen and proteoglycans, the meniscus is predominantly constructed of highly organized collagen type I with a smaller proteoglycan content. The orientation of the collagen fibers within the meniscus is important for its structural characteristics. The meniscus is composed of an extensive network of circumferential, or hoop fibers with penetrating radial fibers throughout. Collagen organization on the articular surfaces of the meniscus is mesh-like to better manage shear stresses. Meniscus collagen content also varies with zones of the meniscus. The inner zone of the meniscus is comprised of a greater proportion of collagen type II, whereas the vascularized outer portion of the meniscus is overwhelmingly collagen type I. Other collagens included in the meniscus include collagens type III, IV, VI, and XVIII. Proteoglycans are also varied by region, but aggrecan is the main proteoglycan.

Meniscus structure and collagen composition differs from cartilage, but the meniscus cell phenotype is also heterogeneous and shares some commonalities. The vascularized outer region of the meniscus is populated by more fibroblastic cells that produce predominantly type I collagen extracellular matrix. The fibrochondrocyte that comprises the avascular inner sections of the meniscus is more similar to articular chondrocytes. Meniscus type I and II collagen production by fibrochondrocytes in the inner region is highly organized and organized differently from articular cartilage. The phenotypic similarities of cartilage and meniscus cells suggest that these cells can have a biologic role in osteoarthritis pathogenesis.

**Osteoarthritis and the Meniscus**

Osteoarthritis continues to be an enigma in joint disease, but it is increasingly apparent that the disease process is a function of whole joint pathology and not solely the articular cartilage. A growing body of evidence supports the connection between a meniscal injury and osteoarthritis. Injuries to the meniscus are commonly evaluated using magnetic
resonance imaging (MRI). MRI is able to detect structural differences and pathologic alterations in tissues based on the proton movement differences in their molecular composition. A large, prospective case-control study identified degenerative meniscus changes using MRI prior to MRI or radiographic evidence of osteoarthritis development\textsuperscript{10}. Additional MRI evaluation of the meniscus demonstrated increased alterations in adjacent regions of meniscus injury and degeneration with concomitant local structural alterations in cartilage\textsuperscript{32, 33}. Meniscus changes evaluated through macroradiography suggested some meniscus alterations preceded cartilage lesions in mild to moderate osteoarthritic knees\textsuperscript{36}. Normal meniscal tissue is rarely found in a knee with osteoarthritis, but knee osteoarthritis may result from a deficient meniscus and may simultaneously serve to further degrade remaining meniscal tissue\textsuperscript{20, 37, 38}. Englund et al. propose that the meniscus tear, especially degenerative tears, initiate the development of osteoarthritis\textsuperscript{18, 19}.

The interactions between the meniscus and articular cartilage are not surprising, since the meniscus and articular cartilage exist in a similar microenvironment and have overlapping gene expression\textsuperscript{7, 17, 31, 39, 40}. The meniscus is exposed to many of the same forces as articular cartilage and responds with some of the same chemical mediators\textsuperscript{17, 31, 39, 41}. Since meniscus cells and articular chondrocytes have some similarities, cartilage pathways involved in osteoarthritis pathogenesis may also be active in meniscal tissue. These molecular pathways may provide an opportunity for intervention immediately following injury, during operative intervention, and during repair to enhance patient quality of life and reduce the burden of osteoarthritis.

\textit{The Role of Pro-inflammatory Factors in the Development of Osteoarthritis}

Early aberrations in cytokine signaling are believed to be responsible for propagating the reactive and degradative responses in joint tissues that ultimately lead to osteoarthritis\textsuperscript{7, 8, 31, 40, 42-50}. Since the knee joint functions as an organ, inflammatory factors produced in acute or chronic injury likely impacts meniscus biology. The impact of cytokine stimulation on articular cartilage
and subsequent extracellular matrix degradation is well documented \(^7, 8, 42, 44, 51\). Treatment of articular chondrocytes with pro-inflammatory factors, such as interleukin (IL)-1\(\alpha\), IL-1\(\beta\), IL-6 and tumor necrosis factor (TNF)-\(\alpha\), results in increased catabolic activity and osteoarthritic changes \(^7, 8, 42, 44, 51-54\). Catabolic pathways include the increased production of the extracellular matrix-degrading matrix metalloproteinases (MMPs), including MMP-1, -3, -9, and -13 in addition to increasing production of cytokines and chemokines \(^7, 8, 43\). Chondrocyte catabolism can also be induced by the cartilage breakdown product, fibronectin fragments (FnF) which are released following chondral insult or injury \(^55\). FnF treatment of chondrocytes results in increased production of MMPs, increased cytokine and chemokine expression, and alterations of integrin expression \(^46, 47, 55-60\). MMPs then degrade components of the extracellular matrix and result in local tissue compromise \(^7, 8, 43\).

Two recent studies utilized a unique approach to examine the differences in gene expression of the entire medial compartment of knee joint. The joint tissues were obtained after destabilized medial meniscus surgery in mice \(^46, 47\). The DMM model is a meniscus injury model known to produce osteoarthritic changes in mice \(^61\). The first study evaluated differences in gene expression between the old and young mice following the DMM surgery using gene microarray and real-time PCR analysis. Many genes with significant and related functions, including those involved in extracellular matrix, signal peptide, leucine-rich repeat, and cytokine activity. Matrix genes included asporin, biglycan, lumican, fibromodulin, periostin (osteoblast specific factor) and procollagens III, VI, and XIV. Asporin, which binds TGF-\(\beta\), is a risk factor for osteoarthritis in humans. MMP-2 and MMP-3, TIMP1, IGF-I and the chemokine CCL21 were also up-regulated in both age groups. Three of the six probe sets that were down-regulated in both young and old mice have significantly related functions of immunoglobulin activity.

The above data are indicative of age-related differences in the severity and pattern of osteoarthritis gene expression in mice. In the second study, a time course experiment was
performed in young mice to examine changes in gene expression at early and late time points after meniscal destabilization. RNA was isolated from the same joint region used in the first study, but samples were obtained multiple early time points following surgery. Gene clusters are again analyzed and demonstrated up-regulation of genes involved in extracellular matrix production, collagen production and cell adhesion. The genes in this cluster were up-regulated at early time points (two and four weeks) during early osteoarthritis development, were relatively unchanged at eight weeks, and were again up-regulated at sixteen weeks. This trend indicates a phasic process in the development of osteoarthritis. Ultimately, these studies support the concept that osteoarthritis is a process of the whole joint, and the meniscus is likely involved in this process.

Many aspects of meniscus biology are pathologically altered in meniscus injury which likely contributes to development of osteoarthritis. Both the biomechanical and cytokine stimulation of porcine meniscus explants increases proteoglycan release, nitric oxide production and also increase intracellular calcium signaling, which is associated with catabolic activity in porcine chondrocytes and meniscus cells. Rabbit meniscus structure and cell phenotype underwent significant alterations during the course of osteoarthritis development following ACL transection. Rabbit menisci in ACL transected joints demonstrated fissuring in the mid-substance of the meniscus with focal cell death in a loss of proteoglycan staining. Cell clusters similar to those in osteoarthritic chondrocyte cell clustering were also identified. With increasing time, fissuring, loss of proteoglycan content, and cell clustering became more severe. Decreased proteoglycan content was accompanied by alterations in collagen deposition. These cell matrix alterations evident on immunohistochemistry were also identified in altered gene expression of extracellular matrix components, including collagen type I, II, and III collagen and aggrecan, in addition to altered MMP expression. The inflammatory genes COX2 and iNOS were up-regulated as well.
To examine zonal differences in the meniscus, sheep menisci were divided into inner and outer zones and subsequently cultured\textsuperscript{31}. The inner zones were more cartilaginous than the outer zones and demonstrated higher expression of aggrecan and collagen 2A1\textsuperscript{31}. Catabolic activity was differentially increased in the two cell types following cytokine stimulation. The outer menisci increased their MMP expression while the inner zones increased ADAMTS5 expression\textsuperscript{31}.

Immunohistochemical analyses of human meniscus specimens support observations in animal studies. Evidence of meniscus injury and degradative enzyme production, specifically MMP-3, aggrecanase-1 and -2, was identified in human immunohistochemistry specimens obtained at the time of partial meniscectomy\textsuperscript{65}. A second study of partial meniscectomy specimens identified increased phosphorylated-p38 (associated with MAP kinase pathway activity) and phosphorylated-p65 (NF-\kappaB pathway activity) in more degenerated and osteoarthritic meniscus specimens\textsuperscript{66}. The increased catabolic pathway staining was associated with an increased in pro-inflammatory cyclooxygenase(Cox)-2 staining\textsuperscript{66}.

Gene expression was also analyzed in human partial meniscectomy specimens obtained during arthroscopy which found increased expression of IL-1\beta, ADAMTS-5, MMP-1, MMP-9, MMP-13, and NFkB2 in patients with meniscus tears younger than 40\textsuperscript{41}. Meniscus cytokine and chemokine expression (including IL-1\beta, TNF-\alpha, MMP-13, CCL3, and CCL3L1) were increased in patients with a more severe injury in patients with a meniscus tear and concomitant ACL tear\textsuperscript{41}. In a human meniscus cell culture study, increased gene expression of MMPs was identified, but these cells were passaged many times and the phenotype may not be consistent with the primary meniscus cell type\textsuperscript{40}. The pathologic alterations in the meniscus support the theory that the meniscus also has a biologic role in osteoarthritis development through the production of matrix-degrading enzymes and inflammatory factors, but further exploration is warranted to define the biologic contributions.
Many of these alterations in chondrocytes are dependent upon the nuclear factor-κB (NF-κB) pathway. The classic NF-κB pathway is comprised of the heterodimer NF-κB1-RelA (also known as the p50-p65 complex). NF-κB then mediates the transcription of pro-inflammatory cytokines, chemokines, adhesion molecules and other catabolic factors. While this pathway is extensively studied in chondrocytes, it is less well studied in the meniscus. The NF-κB subunits p50 and p65 are present in the meniscus and were also demonstrated to be increased in osteoarthritic and degenerative menisci as described above. Many of the alterations in gene expression and protein production are associated with the NF-κB family, so it is likely that meniscus cells also increase catabolic production through stimulation of this pathway.

The Hypoxia Induced Factor Pathway

The hypoxia induced factor (HIF) pathway is implicated in the development of osteoarthritis. HIF-1α is a constitutively expressed protein involved in tissue specific actions. HIF-1α is a transcription factor that serves many anabolic functions by maintaining metabolic homeostasis through cell growth and protection. HIFs are more active in low oxygen tension tissues, such as cartilage and meniscus, because of decreased prolyl hydrolyase domain-containing protein activity (the enzyme responsible for HIF hydroxylation and subsequent degradation); is a critical regulator of glycolytic and oxidation pathways and helps to optimize ATP production and limit reactive oxygen species (ROS) accumulation regardless of the oxygen concentration. HIF-1α increases ATP production in low oxygen tissue through up-regulation of phosphoglycerate kinase 1 (PGK-1), glucose transporter 1 (Glut-1) and vascular endothelial growth factor (VEGF). In articular chondrocytes, HIF-1α is tonically activated with continuous translocation and is reportedly not degraded even when exposed to normoxic conditions in bovine cartilage. Aggrecan and type II collagen are up-regulated via HIF-1α in response to physiologic oxygen tensions in cartilage. Since type II collagen and aggrecan levels are significantly
decreased in HIF-1α-null mutants, HIF-1α was confirmed as a central regulator of glycolysis for generation of the extracellular-matrix.

The role of HIF-1α in energy regulation and matrix production carries important implications for its role in the meniscus. HIF-1α is present in the meniscus with greater expression in the more hypoxic inner regions than the vascularized outer regions of the meniscus. Expression levels varied between meniscus regions, so it is believed that HIF-1α helps to direct meniscus cell phenotype. Although HIF-1α has multiple targets, it is proposed to differentially regulate collagen expression in inner and outer meniscus cells, in part based on oxygen tension. Meniscus cell HIF-1α targets may differ from the targets in chondrocytes which may contribute to the differences in cell phenotype.

A second component of the HIF pathway, HIF-2α, was recently linked to osteoarthritis development in both human and mouse tissues. HIF-2α is encoded by the gene *epas1*. HIF-2α does not appear to be constitutively expressed in a manner similar to HIF-1α in developed tissues; however, in the developing mouse embryo it is critical in the development of multiple organ systems including the musculoskeletal system. Complete deletion of *epas1* in knock-out mice was found to be embryonic lethal; however, heterozygous deletion of *epas1* in *epas1*+/− transgenics provided resistance to surgically induced osteoarthritic changes using the destabilized medial meniscus (DMM) model. The mechanism for resistance was not explored and remains unclear. Transgenic mice over-expressing *epas1* had increased osteoarthritic changes in the knee cartilage. Levels of HIF-2α were additionally increased in osteoarthritic cartilage of the DMM mice. Similar trends were seen in human osteoarthritic cartilage: levels of HIF-2α and its targets were present in damaged human osteoarthritic cartilage, while levels were lower in normal and undamaged osteoarthritic cartilage compared to damaged regions of the osteoarthritic cartilage. HIF-2α expression in the meniscus was not investigated in any study.
Multiple pro-inflammatory cytokines, including TNF-α, IL-1β, IL-17 and IL-21, induce HIF-2α expression but expression is not increased with anti-inflammatory cytokines (IL-4, IL-10, IL-12) \(^{79, 85, 86}\). Unlike HIF-1α activity which is better regulated by oxygen tension, HIF-2α activity is reported to be independent of oxygen concentration and is instead regulated by increasing its expression level \(^{79, 86}\). Signaling of HIF-2α involves the c-Jun-terminal kinase (JNK) and NF-κB signaling pathways \(^{79, 86}\). The Epas1 gene contains an NF-κB binding motif recognized by p65/RelA, a subunit of NF-κB. Activation of p65/RelA was significantly increased in osteoarthritic tissue, which paralleled the increase in HIF-2α activity \(^{79, 86}\). NF-κB has a prominent role in mediating the inflammatory response within cartilage from mechanical signal transduction \(^{66, 71}\). The activation of the NF-κB pathway in the meniscus is directly linked to fibrocartilaginous degeneration and osteoarthritis \(^{66}\). HIF-2α and NF-κB are also both involved in the complex regulation of sox9 expression and extracellular matrix production, which are both implicated in chondrocyte differentiation and degradation \(^{66, 85, 93-95}\).

While the exact role of HIF-2α up-regulation in cartilage degradation is not established, a number of targets have been discovered that link it to ECM changes. Recently discovered targets of HIF-2α include COL10A1, MMP1, MMP3, MMP9, MMP12, MMP13, VEGF-A, ADAMTS4, NOS2 and PTGS2 in chondrocytes \(^{79, 85, 86}\). The authors attributed the primary deleterious effects in cartilage to MMP9 expression, although it is likely a component in a series of inappropriate regulatory events. Additional studies of HIF-2α in mouse and human osteoarthritis cell cultures identified its role in regulating the pro-inflammatory cytokine IL-6 production and articular chondrocyte apoptosis \(^{96, 97}\). Expression of many of these genes increases following meniscus injury in the rabbit and is associated with meniscus injury and osteoarthritis in humans \(^{40, 41, 64, 70}\). Since HIF-1α is expressed in the meniscus and many gene targets of HIF-2α are present in the meniscus, expression of HIF-2α is likely present in the meniscus.
HIF-2α expression appears to target catabolic expression in some studies, but the relationship of HIF-1α was not explored in the recent studies in mice\textsuperscript{79, 86}. An appropriate balance between the effects of HIF-1α and HIF-2α is likely more important than either gene in isolation. Support for this balance is found in the association of HIF-2α with some anabolic processes and finding that human chondrocytes may respond differently than mouse chondrocytes\textsuperscript{81, 82, 98}. Increased HIF-2α in human chondrocytes is also linked to increased SOX9 expression and increased expression of chondrocytic extracellular matrix genes: COL2A1, COL9A1, COL11A2 and AGGREGAN\textsuperscript{82, 91}. Additionally, HIF levels can be heavily influenced by prolyl hydroxylation as demonstrated in some studies\textsuperscript{81, 82, 98}, and may not be stabilized by inflammatory stimulation to the degree originally proposed in recent studies\textsuperscript{79, 86}. In larger scale human analysis, a functional single nucleotide polymorphism in the human EPAS1 gene (HIF-2 α) was linked to osteoarthritis in a Japanese population to but, a second study with a larger cohort found conflicting evidence that did not support their conclusion\textsuperscript{99, 100}. A third component of the HIF pathway, HIF-3α, was also recently implicated in osteoarthritis and may additionally regulate HIF-1α and HIF-2α\textsuperscript{101}. The authors identified that proteoglycan 4 (Prg4) upregulated HIF-3α, which in turn, down-regulated HIF-1α and HIF-2α in mice. Suppression of chondrocyte hypertrophy and catabolic activity was suggested by the findings that overexpression of Prg4 resulted in increased HIF-3α expression with decreased VEGF, collagen type X, and MMP13 expression\textsuperscript{101}. The potential role of HIF-3α in protecting against catabolic responses is intriguing, but additional exploration is warranted since HIF-3α was reported as minimally expressed in chondrogenic differentiation of mouse ATDC5 cells\textsuperscript{86}. In light of this conflicting evidence, it is important to examine the balance of HIF expression and their target gene response to better understand this novel pathway in cartilage and in meniscus cells. Furthermore, it is important that these evaluations be performed in human cells to determine if the human meniscus cell behaves similarly to the described human chondrocytes and mouse chondrocytes. The coregulation of the HIF pathway in meniscus cells is
an opportunity to explore what appears to be a dynamic pathway in joint tissues protection and degradation.

**Exploring the biologic contribution of the meniscus in osteoarthritis pathogenesis**

The overarching goal of this research is to identify the biologic role of the meniscus in the development of osteoarthritis. We hypothesize that inflammatory factors associated with joint injury would stimulate menisci to increase production of matrix degrading enzymes, cytokines and chemokines which could contribute to joint tissue destruction and subsequent development of osteoarthritis. We will examine meniscus pathology in developed osteoarthritis, characterize the alterations in meniscus cell processes between normal and osteoarthritic menisci, and explore potential pathways involved in osteoarthritis pathogenesis including a novel pathway proposed to mediate osteoarthritis development.

The first objective of the study is to determine whether the degradative changes in the meniscus coincide with degradation of cartilage in the development of osteoarthritis. Vervet monkeys will be used to examine meniscus pathology as part the natural history of osteoarthritis. Non-human primates are known to develop osteoarthritis both spontaneously and with surgical models\(^{102-106}\). A pilot project at our institution identified osteoarthritic changes in a well characterized colony of vervet monkeys\(^{106}\). A cross-sectional analysis of two cohorts of young and old vervets will be evaluated with radiographic studies and gross pathology to determine evidence of osteoarthritis development. The meniscus will then be examined for biologic activity by analyzing protein secretion.

The second objective will be to explore differences in response in normal and osteoarthritic meniscus cells by evaluating key regulatory signaling implicated in osteoarthritis pathogenesis. Human meniscus cells will be collected from normal tissue donors in patients undergoing total knee arthroplasty to examine their response to pro-inflammatory stimulation through extracellular
matrix related gene expression and protein production. Recent evidence is more similar pathways in the meniscus nursing cartilage, will explore the target genes associated with the NF-κB pathway. These genes include extracellular matrix related genes in addition to cytokine and chemokine expression that was previously identified in the meniscus. It is expected that the NF-κB pathway will be activated during disease progression.

The third and final objective of this research will be to evaluate role of the HIF pathway that may contribute to osteoarthritis pathogenesis in human meniscus cells and evaluate them in the context of a mouse surgical model of osteoarthritis. HIF-1α is present in both the human and mouse meniscus. Likewise, it is expected that HIF-2α is also expressed in meniscal tissue. It appears that the balance in HIF-1α and HIF-2α activity controls genes that regulate matrix synthesis and degradation. An inappropriate up-regulation of HIF-2α in the articular cartilage has been linked to osteoarthritis development in mice and humans, so it is plausible that similar patterns may be observed in meniscus cells in response to injury. An effective strategy to identify these alterations is to first demonstrate HIF-1α and HIF-2α in the meniscus and then to evaluate changes in relevant gene expression with over-expression and down regulation of HIF-1α and HIF-2α. The degree of osteoarthritic changes in the mouse meniscus after injury in a HIF-2α +/- knock-out will be used to confirm the role of this protein in the development of osteoarthritis. The focus on the early development of osteoarthritic changes in mice and examination of human osteoarthritic tissue may identify early molecular alterations in the HIF pathway and related gene expression. These signaling pathways may then represent important therapeutic targets in the meniscus that when inhibited could prevent or attenuate the meniscal degeneration that contributes to the subsequent development of osteoarthritis. Furthermore, HIF influence on chondrocyte differentiation may allow for alteration of cell phenotype in tissue engineering applications of the heterozygous meniscus tissue.
References


75. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) 2009; 2497-106.


77. Duval E, Leclercq S, Elissalde J-M, Demoor M, Galéra P, Boumédiene K. Hypoxia-inducible factor 1α inhibits the fibroblast-like markers type I and type III collagen during hypoxia-induced chondrocyte redifferentiation: Hypoxia not only induces type II collagen and aggrecan, but it also inhibits type I and type III collagen in the hypoxia-inducible factor 1α–dependent redifferentiation of chondrocytes. Arthritis & Rheumatism 2009; 60(10): 3038-3048.


91. Lafont JE, Talma S, Murphy CL. Hypoxia-inducible factor 2α is essential for hypoxic induction of the human articular chondrocyte phenotype. Arthritis & Rheumatism 2007; 56(10): 3297-3306.


Osteoarthritic changes in the vervet monkey knees correlate with meniscus degradation and increased MMP and cytokine production.

Austin V. Stone MD, Richard F. Loeser MD, David L. Long, John R. Stehle Jr PhD,

Thomas C. Register PhD, Cristin M. Ferguson MD
Abstract

Objective: Meniscus injury increases osteoarthritis risk but its pathobiology in osteoarthritis is unclear. We hypothesized that degenerative menisci would secrete increased matrix metalloproteinases (MMPs) and pro-inflammatory cytokines.

Methods: In a cross sectional analysis of healthy middle-aged and old vervet monkeys, knees were morphologically graded and evaluated with computed tomography (CT) imaging. Meniscus explants were subsequently cultured to analyze MMP and cytokine secretion.

Results: Meniscus and cartilage degradation scores were positively correlated \((r>0.7)\). CT images revealed osteoarthritic changes in 80% of old monkeys and these changes corresponded to higher grade morphologic lesions. Osteoarthritic monkey menisci secreted significantly greater more MMP-1, MMP-3, and MMP-8 than healthy menisci. Older menisci produced increased IL-6 and IL-7, while older osteoarthritic menisci also secreted more granulocyte-macrophage colony-stimulating factor (GM-CSF) than healthy and middle-aged menisci.

Conclusion: Aged and degenerative menisci produced increased amounts of matrix-degrading enzymes and inflammatory cytokines. Secreted proteins and enzymes would be expected to act both on both the meniscus tissue and local knee tissues, which may degrade the extracellular matrix and propagate inflammatory cycles. The dynamic relationship between menisci and cartilage likely extends beyond biomechanical involvement to biologic interactions between the tissues.
Introduction:

Osteoarthritis is increasingly recognized as a disease of the whole joint with a shared environment comprised of cartilage, synovium, ligaments and the meniscus\(^1\). The impact of cytokine stimulation on articular cartilage and subsequent extracellular matrix degradation is well documented\(^2,3\); however, the role of the meniscus in this process is unclear. Meniscus injury is a known predisposing factor for osteoarthritis, and meniscus biology is likely impacted by exposure to the inflammatory factors produced by knee tissues in response to acute or chronic injury. Certain aspects of meniscus biology are pathologically altered in meniscus injury and in the development of osteoarthritis\(^4-6\). Thus, the meniscus likely also has a biologic role in osteoarthritis development. Release of matrix metalloproteinases (MMPs) and pro-inflammatory factors from the meniscus could negatively affect the nearby articular cartilage and synovium. We hypothesized that aged and damaged menisci would produce catabolic factors, including MMPs and pro-inflammatory cytokines, which may contribute to the development of osteoarthritis.

Material and Methods:

All animal procedures were approved and in accordance with federal and institutional animal care and use guidelines. Five middle-aged (9.3-11.6 years) and five old (19.7-26.2 years) healthy adult female African green vervet (Chlorocebus aethiops sabaeus) monkeys were included. These monkeys were part of a unique vervet colony raised in social groups to reflect life in the wild. Whole body computed tomography (CT) scans were obtained (Toshiba Aquilon 32 Slice CT) and analyzed with CT slices and reconstructions using AquariusNet (TeraRecon, Inc.).

Vervets were euthanized and harvested knees were disarticulated and graded for cartilage and meniscus degradation using the International Cartilage Repair Society scoring (http://www.cartilage.org/index.php?pid=223) with a meniscus adaption. Meniscus specimens were individually cultured as whole explants in 12 well culture plates in DMEM/F12 media (Gibco) with 10% fetal bovine serum (FBS; Gibco). After overnight acclimation, explants were
changed to serum-free media and cultured for 48 hours. Conditioned explant media was then collected and target proteins were analyzed.

For protein analysis, equal volumes of conditioned media were separated by SDS-PAGE (BioRad), transferred to nitrocellulose (Odyssey, Invitrogen) and probed with the primary antibody [anti-MMP1 (PAB12708, Abnova); anti-MMP3 (AB2963, Millipore); anti-MMP8 (MAB3316, Millipore); anti-MMP13 (AB84594, Abcam)] and secondary antibody (CellSignal). Blots were visualized with chemiluminescence (Amersham ECL, GE Life Sciences). Media was analyzed with an MMP protein array (#AAH-MMP-1, RayBiotech). Conditioned media was also analyzed with a cytokine array (#AAH-CYT-1; RayBiotech) according to the manufacturer’s protocol. Processed films were imported into Photoshop v7.0 (Adobe) and labeled. Densitometry measurements were completed with ImageJ 1.44p (NIH) and normalized to explant weight.

Statistical analysis was performed with SigmaPlot v10.0 (Systat Software) and Prism v5.02 (GraphPad Software, Inc.). Vervet knee morphology scores were analyzed using Spearman rank correlation while densitometry data was analyzed using two way analysis of variance (ANOVA) with post-hoc Tukey Tests. Significance was set at $\alpha \leq 0.05$.

**Results:**

Tabulated vervet knee morphologic scores are outlined in Figure 1 (complete data set in Table S1). Cartilage and medial meniscus scores correlated with age (right $r=0.77$, $p=0.007$; left $r=0.73$, $p=0.013$). Meniscus degradation correlated with femoral and tibial cartilage degradation in the respective medial ($r \geq 0.79$, $p \leq 0.005$) and lateral compartments ($r \geq 0.63$, $p \leq 0.048$). Meniscus degradation scores were significantly greater in old versus young vervets in both compartments ($p=0.002$). Medial and lateral compartment cartilage degradation was significantly greater in old vervets ($p<0.001$). Degradation scores of the medial meniscus correlated with the lateral cartilage
scores ($p<0.001$) and no significant difference existed between medial and lateral meniscus scores.

CT scans demonstrated osteoarthritic changes in aged joints. Middle-aged vervets did not exhibit significant osteoarthritic changes, but 80% (4/5) of the old vervets had osteophyte formation and evidence of subchondral bone cyst formation (Figure 1). In vervets with osteoarthritic changes, medial compartment scores were consistently high, but lateral compartment scores were more variable.

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Young (9-12 yrs) approximately 30 human yrs

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Old (20-26 yrs) approximately 70 human yrs

Figure 1: Young and old vervet knee CT scans with morphologic assessment.

Morphology scores (Table 2) were tabulated for each limb as a measure of disease severity. Scores are reported above the CT scans. CT reconstructions from young (9.3-11.6 years) and older (19.7-26.2 years) vervet knees.

Vervet menisci were cultured as explants to assess MMP production and cytokine production. Compared to explants from young animals, osteoarthritic meniscus explants from older animals demonstrated increased production of MMP-1, -3, and -8 (respectively $p=0.0192$, $p=0.0483$, $p=0.0495$; Figure 2). Medial menisci produced greater amounts of MMP-1 and -8 than the lateral meniscus, which paralleled the higher grade degenerative changes.
Figure 2: MMP protein secretion in young and old vervet menisci with densitometric analysis. A) Representative immunoblots of conditioned media from meniscus explant culture. MMP-1 and MMP-8 and MMP-3. [Right (R) or left (L) modified with medial (M) or lateral (L)]. B) Densitometric analysis of MMP blots. Each data point is the mean response of the meniscus from each compartment (right or left knee and medial or lateral compartments; n=3 unique donors) and the line is the mean for all young or old knee compartments. Densitometry units were normalized to explant tissue weight. MMP-1: p= 0.0192; MMP-3: p= 0.0483; MMP-8: p=0.0495. Error bars are standard error of the mean.

Inflammatory cytokine production in meniscal explant media was also analyzed. Explants from both young and old animals produced IL-8, and GRO family chemokines (GRO antibody on the array binds CXCL1(GROα), CXCL2(GROβ), CXCL3(GROγ); Figure 3, n=1); older menisci demonstrated a slight increase in IL-6 production but a substantial increase in IL-7.
The higher morphologic grade medial meniscus also secreted granulocyte-macrophage colony-stimulating factor (GM-CSF).

**Figure 3:** Cytokine protein array from young and old monkey menisci. Conditioned media from meniscal explant cultures was incubated with cytokine protein array membranes. (+ positive control; G0: grade 0; G3: grade 3)

**Discussion:**

The clinical importance of the meniscus in osteoarthritis development is well documented\(^7\); however, meniscus pathology in osteoarthritis is largely attributed to mechanically mediated loss of structural integrity\(^8\). These biomechanical stress factors may lead to “osteoarthritis in the meniscus” which is proposed to be responsible for MRI changes seen in the meniscus during the early development of osteoarthritis\(^8\). Recent evidence suggests the meniscus may have a much more biologically active role in the whole joint pathology in osteoarthritis\(^5,6\). Our data support...
previous gene expression reports and identifies biologic contributions from meniscus involvement implicated in osteoarthritis pathogenesis.

Vervet knees were demonstrated to have osteoarthritic changes that were similar to humans, and consistent with established reports of spontaneous and surgically induced osteoarthritis in non-human primates\textsuperscript{9-13}. Osteoarthritic changes were previously identified in the vervet shoulder which included glenoid retroversion and joint space narrowing\textsuperscript{13}. Our cross sectional analysis suggested that pathologic degradation of the meniscus corresponded to more severe bony changes and cartilage degradation. Meniscus degenerative changes were previously correlated with osteoarthritic changes in cartilage and articular cartilage loss\textsuperscript{7, 14, 15}. In a small human magnetic resonance imaging (MRI) study, meniscus deterioration accompanied or preceded severe articular cartilage loss\textsuperscript{14}. A larger MRI study identified an increasing risk of cartilage loss with increasing number of meniscus abnormalities\textsuperscript{15}. We identified similar correlations between meniscus and cartilage morphologic scores and found that higher degeneration scores were associated with a greater production of matrix metalloproteases (MMPs) and cytokines.

Early aberrations in cytokine signaling are believed to be responsible for propagating the reactive and degenerative responses in bone and cartilage that ultimately lead to osteoarthritis\textsuperscript{2, 3, 16}. The increase in MMP-1 production is important because it degrades collagen type I, which is the primary constituent of meniscal extracellular matrix. Increased MMP-3 (stromelysin-1) production is similarly important because it is upregulated in articular cartilage in early osteoarthritis\textsuperscript{3, 16}. MMP-8 activity in osteoarthritis pathogenesis is less well studied, but it is capable of degrading collagen, and is associated with neutrophil infiltration, and co-localizes with IL-1\(\beta\) and type II collagen cleavage in osteoarthritic cartilage\textsuperscript{3}. Comparable pathways are activated in the pig meniscus. Pig meniscus explants demonstrated increased MMP1 activity, proteoglycan release and nitric oxide release in response with IL-1 stimulation\textsuperscript{17}. 
Meniscus cells can be stimulated to produce matrix degrading enzymes which can impact cartilage, but the tissue interaction is likely part of a more dynamic signaling network. We identified age and disease dependent cytokine production by meniscus explants. Chemokines CXCL1, CXCL2, CXCL3 (identified by the GRO antibody) and IL-8 were expressed in both young and old menisci which may not independently initiate osteoarthritic changes but may contribute to the development of inappropriate inflammatory cycles after injury\(^1,3\). These genes were recently identified as part of the disease process in meniscus injury\(^4,5\). GM-CSF was up-regulated in more degenerative meniscus. GM-CSF is linked to the inflammatory process in rheumatoid arthritis but was also reported in osteoarthritis synovium\(^18\). Future investigation may link GM-CSF production to the more fibroblastic cell phenotype in the meniscus. IL-6 secretion appeared to be elevated in older and higher grade vervet menisci but was also present in younger vervet menisci. Increased IL-6 production is implicated in osteoarthritis pathogenesis\(^1,3\). The reason for elevated vervet IL-6 secretion is unclear, but IL-6 production in younger vervet menisci could be evidence of early pathology. IL-7 was also shown to be produced by aged and degenerative vervet menisci. IL-7 stimulates human chondrocyte MMP-13 production, extracellular matrix degradation, proteoglycan release from human cartilage explants\(^2\).

Chondrocytes not only respond to IL-7 by increases in MMP-13 production, but they may be stimulated to produce IL-7 by IL-1 and IL-6. Additionally, IL-7 is reportedly higher in humans during earlier stages of osteoarthritis and is also elevated in older patients with osteoarthritis and patients with synovitis\(^1,19\). IL-8 is thought to contribute to chondrocyte hypertrophy, calcification and crystals in the joint, and the development of subchondral bone sclerosis\(^1\).

Our results confirm many of the trends recently reported in an age and sex analysis of gene expression in meniscus tears\(^5\). We observed both age and disease related changes in meniscus protein production. Cytokine and matrix degrading enzyme production can be assumed to act on local tissues. In the case of the meniscus, the secretion of enzymes and cytokines are likely
acting on both cartilage and the synovium to stimulate catabolic processes. This concept may be one way the meniscus contributes to osteoarthritis development, especially after meniscal injury. Additionally, these findings may better explain the higher failures in meniscus repair in older patients. We observed age related increases in inflammatory cytokines and MMP production in the natural history of osteoarthritis in monkeys. Older patients with a previous meniscus injury are likely producing increased matrix degrading enzymes as a function of both the initial injury and age, and both factors are likely to contribute to disease progression.

Our study carries common limitations of laboratory and animal models. Although we analyzed the monkey CT slices and reconstructions, we cannot directly compare the scans to human radiographs; however, we believe the monkey radiographic and biologic findings are clinically relevant to the human disease process. In this preliminary analysis, whole explants were used to assess the contribution of both the inner and outer zones of the meniscus to protein secretion that would be expected in the joint. This study sought to identify catabolic patterns in menisci and place them in the context of the natural disease progression in a monkey model. Future studies will examine osteoarthritis pathobiology of the meniscus in greater detail.

The role of the meniscus in osteoarthritis pathogenesis remains to be defined, and this study sought to better understand the biologic activity of the meniscus in osteoarthritis. The production of matrix degrading enzymes correlated with morphologic changes in osteoarthritic monkey knees. While the full cell mechanism was not explored, we believe that the increased expression of MMPs and cytokines as response to inflammatory cytokines likely plays a role in osteoarthritis pathogenesis in the meniscus and articular cartilage. The ultimate goal of this research is to treat early pathology in an effort to prevent, or at least attenuate, the development of osteoarthritis.
Funding Source

This work was funded by grants from the Orthopaedic Research and Education Foundation and the American Orthopaedic Society for Sports Medicine. Support was also received from a NIH/NIAMS (K08AR059172) and NIH/NIA (A6044034). Vervet monkey specimens were obtained from the Wake Forest Primate Center and with funding from the NIH (NCRR-P40 RR019963), Department of Veterans Affairs (VA 247-P-0447) and the Wake Forest University Claude D. Pepper Older Americans Independence Center (P30 AG021332).
References


Supplementary Material

Table S1: Vervet knee cartilage and meniscus scores.

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Pro-inflammatory stimulation of meniscus cells increases production of matrix metalloproteinases and additional catabolic factors involved in osteoarthritis pathogenesis.

Austin V. Stone MD, Richard F. Loeser MD, David L. Long, Kadie S. Vanderman MS,

Stephanie C. Clark, Cristin M. Ferguson MD
Abstract

Objective: Meniscus injury increases the risk of osteoarthritis; however, the biologic mechanism remains unknown. We hypothesized that pro-inflammatory stimulation of meniscus would increase production of matrix-degrading enzymes, cytokines and chemokines which cause joint tissue destruction and could contribute to osteoarthritis development.

Design: Meniscus and cartilage tissue from healthy tissue donors and total knee arthroplasties was cultured. Primary cell cultures were stimulated with pro-inflammatory factors [IL-1β, IL-6, or fibronectin fragments (FnF)] and cellular responses were analyzed by real-time PCR, protein arrays and immunoblots. To determine if NF-κB was required for MMP production, meniscus cultures were treated with inflammatory factors with and without the NF-κB inhibitor, hypoestoxide.

Results: Normal and osteoarthritic meniscus cells increased their MMP secretion in response to stimulation, but specific patterns emerged that were unique to each stimulus with the greatest number of MMPs expressed in response to FnF. Meniscus collagen and connective tissue growth factor gene expression was reduced. Expression of cytokines (IL-1α, IL-1β, IL-6), chemokines (IL-8, CXCL1, CXCL2, CSF1) and components of the NF-κB and tumor necrosis factor (TNF) family were significantly increased. Cytokine and chemokine protein production was also increased by stimulation. When primary cell cultures were treated with hypoestoxide in conjunction with pro-inflammatory stimulation, p65 activation was reduced as were MMP-1 and MMP-3 production.
Conclusions: Pro-inflammatory stimulation of meniscus cells increased matrix metalloproteinase production and catabolic gene expression. The meniscus could have an active biologic role in osteoarthritis development following joint injury through increased production of cytokines, chemokines, and matrix-degrading enzymes.

Key Words: meniscus, osteoarthritis, cytokine, matrix-metalloproteinase, MMP
**Introduction**

Meniscus injury is known to increase the risk of osteoarthritis. Untreated meniscus tears have an odds ratio of 5.7 for the development of radiographic osteoarthritis\(^1\). Even after partial meniscectomy, the relative risk (RR) for osteoarthritis increases following both degenerative tears (RR 7.0) and traumatic tears (RR 2.7)\(^2,3\). Successful repairs may lead to resumption of sports activity and decreased incidence of osteoarthritis\(^4\); however, many tears are not amenable to repair secondary to the tissue’s minimal vasculature. This increased risk is historically attributed to changes in knee biomechanics due to meniscus deficiency\(^3,5,6\).

The impact of cytokine stimulation on articular cartilage and subsequent extracellular matrix degradation is well documented\(^7,9\); however, the role of the meniscus in this process is unclear. The knee joint functions as an organ with a shared environment comprised of cartilage, synovium, ligaments and the meniscus. The meniscus is consequently exposed to inflammatory factors produced by knee tissues in response to acute or chronic injury and this exposure likely impacts meniscus biology. Certain aspects of meniscus biology are pathologically altered in meniscus injury and in the development of osteoarthritis\(^10-18\). Thus, the meniscus likely also has a biologic role in osteoarthritis development through the production of matrix-degrading enzymes and inflammatory factors. We hypothesized that inflammatory factors associated with joint injury would stimulate menisci to increase production of matrix degrading enzymes, cytokines and chemokines which could contribute to joint tissue destruction and subsequent development of osteoarthritis.
Materials and Methods

Knee Tissue Acquisition: Our institutional review board approved this protocol. Normal human meniscus specimens (n=14 menisci from n=14 donors 25-58 years old) were procured through the National Disease Research Interchange (NDRI, Philadelphia, PA) or the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) while osteoarthritic menisci were obtained from patients undergoing total knee arthroplasty (TKA) for osteoarthritis (n=31 menisci from n=31 donors 44-83 years old). Synovial tissue was removed. Meniscus tissue was macroscopically graded according to a modified International Cartilage Research Society Cartilage Morphology Score (Table S1). All normal meniscus specimens were a grade zero or one, while all but one osteoarthritic meniscus was a grade three or four (one osteoarthritic meniscus received a morphology grade two). Articular cartilage from TKA bone cuts was processed as above. All comparisons between chondrocytes and meniscus cells used tissue from the same donor.

Cell culture: Normal and osteoarthritic human meniscus and articular chondrocytes were isolated using our laboratory’s tissue digestion and processing methods and primary cells cultured to confluence as described. Prior to stimulation, primary cultures were incubated overnight in serum-free media (DMEM/F12) and then treated for either 6 or 24 hours with one of the following: 10 ng/ml IL-1β; 10 ng/ml IL-6 with 25ng/ml soluble IL-6 receptor; or TGF-α 20ng/ml (all from R and D Systems) or fibronectin fragments (FnF), a recombinant fragment of fibronectin protein containing domains 7-10 of full length fibronectin (at 1 μM; gift from Harold Erickson, Duke University). For NF-κB studies, cells were stimulated as above for 30 minutes with or without the NF-κB inhibitor,
hypoestoxide (25 μM, Sigma), and then lysates were collected. Media was collected for MMP analysis and cells were harvested by scraping in either Trizol (Invitrogen) for RNA isolation or lysis buffer [lysis buffer (Cell Signal Technologies) plus Phosphatase Inhibitor Cocktail 2 (Sigma) and phenylmethanesulfonyl fluoride (Sigma)] for protein analysis.

**Gene and Protein Analysis:** RNA was quantified (Nanodrop, ThermoScientific) and verified (BioAnalyzer Chip, Agilent) to ensure high quality RNA (RIN > 6). The reverse-transcription PCR generated cDNA (RetroScript Kit, Ambion). Real-Time PCR was performed using the Applied Biosystems 7900HT thermocycler with TaqMan Universal PCR MasterMix and Taqman Gene Assay (Applied Biosystems: mmp1 Hs00899658_m1; mmp3 Hs00968305_m1; GAPDH Hs02758991_g1). Data was analyzed using the ΔΔCT method in Microsoft Excel (Microsoft).

For quantitative real-time PCR arrays, RNA was harvested as above and purified using the RNEasy Mini kit (Qiagen, #74104). The purified RNA was then used for the extracellular matrix and adhesion PCR array (SABiosciences, #PAHS-013ZA-12) or NF-κB target gene PCR array (SABiosciences, #PAHS-225ZA-12) and the manufacturer’s optimized master-mix (SABiosciences, #330522) for the Applied Biosystems 7900HT thermocycler according to the manufacturer’s protocol.

For protein analysis, cell media was loaded in equal volumes (1:1 in Lamelli Sample Buffer, 5% β-mercaptoethanol; BioRad), separated by SDS-PAGE (BioRad), transferred to nitrocellulose (Odyssey, Invitrogen) and probed with the primary antibody [anti-MMP1 (PAB12708, Abnova); anti-MMP3 (AB2963, Millipore); anti-MMP8 (MAB3316,
Millipore); anti-MMP13 (AB84594, Abcam)] and secondary antibody (CellSignal).

Blots were visualized with chemiluminescence (Amersham ECL, GE Life Sciences).

Since no known control exists for meniscus secreted proteins, loading was controlled by loading an equal volume of media from wells that had equivalent cell numbers verified by total protein content. Media was analyzed with an MMP Protein Array (#AAH-MMP-1, RayBiotech) or the Cytokine Array (#AAH-CYT-5, RayBiotech). For the NF-κB experiments, immunoblots were probed for phosphorylated p65, then stripped and probed for total p65, and then finally β-actin as the loading control. Processed films were imported into Photoshop v7.0 (Adobe) and labeled. Densitometry was completed with ImageJ 1.44p (NIH).

Statistical Analysis: Statistical analysis was performed with SigmaPlot v10.0 (Systat Software) and Prism v5.02 (GraphPad Software, Inc.). Real-time PCR arrays were analyzed in Microsoft Excel (Microsoft) using the standard ΔΔCt method normalized to endogenous housekeeping genes in array-specific analysis templates (SABiosciences, http://www.sabiosciences.com/pcrarraydataanalysis.php). The template employed the Student t-test for replicates of four individual donors with significance of p ≤ 0.05. We accepted this analysis method with the understanding that we did not account for multiple comparisons. It is possible that a small number of genes may be significantly different because of the number of genes analyzed. This limitation was accepted because we chose to analyze related genes of either extracellular matrix proteins or the NF-κB family.

Additional gene expression experiments were analyzed with two-way analysis of variance (ANOVA). An ANOVA on ranks with post-hoc Holm-Sidak tests was used where appropriate when the data did not meet Gaussian distribution and homogenous
variance. Comparisons between osteoarthritic and normal meniscus gene expression were performed using a three-way ANOVA. The Holm–Sidak test was used for multiple comparisons testing when group effects were found to be significant. We reported Bonferroni corrections for multiple comparisons. Immunoblot densitometry was analyzed using ANOVAs. Significance was set at \( p \leq 0.05 \).

**Results**

**Response of normal meniscus to pro-inflammatory factors**

Normal meniscus cell cultures were stimulated with pro-inflammatory factors to evaluate alterations in extracellular matrix gene expression. Meniscus cells were stimulated with IL-1\( \beta \), IL-6, or fibronectin fragments (FnF). FnFs are found in the synovial fluid and extracellular matrix of arthritic joints and are known to induce cartilage degradation but have not been studied with meniscus\(^{19-22}\). The pro-inflammatory stimuli significantly increased expression of multiple matrix-degrading enzymes, including many of the primary MMPs responsible for degradation of both meniscus and cartilage matrix; however, the specific MMPs expressed varied according to the stimulus (Table 1). All three stimuli increased expression of MMP-1 while IL-1\( \beta \) also stimulated MMP-2 and MMP-10 expression and IL-6 stimulated MMP-3 and ADAMTS1 expression. FnF produced the most significant increase in MMP-1 as well as MMP-2, MMP-3, MMP-8, MMP-10, and MMP-13. FnF stimulated expression of the cell adhesion molecules VCAM-1 and \( \alpha_1 \)- and \( \alpha_2 \)-integrins, while IL-1\( \beta \) stimulated \( \alpha_1 \)- and \( \beta_1 \)-integrin expression (Table 1). IL-6 uniquely stimulated \( \beta_2 \)-integrin expression. Matrix proteins decreased by FnF include collagen VI\( \alpha_1 \), versican, thrombospondins-1 and -3 and connective tissue growth factor (CTGF; Table 1) while collagen VII\( \alpha_1 \) and
lamininβ3 were increased. In contrast, IL-1β increased expression of catenins including α1, β1, and δ2 as well as hyaluronan synthase-1 which was also increased by FnF. IL-6 uniquely down-regulated collagen XVIα1 and versican and similar to FnF decreased thrombospondin-1. Genes on the array which did not have a significant change in response are shown in Table S2.

After identifying alterations in extracellular matrix gene expression, we examined changes in expression and production of selected MMPs that could be secreted and cause local tissue destruction. For this set of experiments, we also included stimulation with TGF-α. TGF-α is a less well studied cytokine in osteoarthritis pathogenesis, but is implicated in articular cartilage degradation\textsuperscript{23,24}. Cytokine stimulation significantly increased mean MMP-1 gene expression in meniscus cultures (Figure 1A, p=0.004). MMP-1 gene expression was significantly greater at 24 hours than six hours (p=0.018). MMP-1 expression was significantly increased by IL-1β (p=0.017), IL-6 (p=0.021), and FnF (p<0.001). Mean MMP-3 gene expression also significantly increased following cytokine stimulation (Figure 1A, p=0.022). Mean gene expression at 24 hours was significantly greater than six hours (p=0.007). IL-1β (p=0.003) and FnF (p=0.001) significantly increased MMP-3 expression.

Protein production of selected MMPs was evaluated by immunoblot. The first set of primary meniscus cell cultures were stimulated with IL-1β, IL-6, or TGF-α. Meniscus cells significantly increased MMP-1 production following stimulation by IL-1β (18.3±9.70 fold), IL-6 (24.1±1.18 fold), and TGF-α (5.78±1.47 fold) (Figure 1B, p=0.0091). MMP-3 was also significantly increased by stimulation with IL-1β (5.24±2.45 fold), IL-6 (3.70±1.31 fold), and TGF-α (2.46±0.96 fold) (Figure 1B, p=0.0207);
MMP-2 was used as a gel loading control since its secretion was similar to the unstimulated controls.

Similar to the first set of experiments, FnF treated meniscus cultures exhibited increased MMP-1 and MMP-3 (Figure 1B). MMP-1 production significantly increased in response to IL-1β, IL-6 and FnF stimulation with respective fold increases of 17.1±12.2, 21.41±10.0, and 26.7%±11.2 (Figure 1B, p=0.032). Stimulation increased MMP-3 as well: IL-1β, 2.76±0.56 fold; IL-6, 3.41±0.91 fold; and FnF, 3.45±0.89 fold (Figure 1B, p=0.027). Normal meniscus cells also produced some MMP-13 in response to IL-6 in particular; however, the response was variable and densitometry did not reach statistical significance (p=0.095).

**Response of osteoarthritic meniscus to pro-inflammatory factors**

Osteoarthritic meniscus cells were also stimulated with pro-inflammatory cytokines to evaluate alterations in MMP expression (Figure 2). MMP-1 expression increased in response to pro-inflammatory cytokine stimulation; however, this response only trended toward significance (p=0.080). This increase appeared greater by 24 hours, but again was not significant (p=0.060). MMP-3 expression responded similarly to MMP-1. MMP-3 expression was greater at 24 hours compared to six hours (p=0.013).

MMP-1 and MMP-3 expression in normal and osteoarthritic meniscus cultures in response to IL-1β, IL-6, and TGF-α was compared to evaluate disease differences using a three way ANOVA. Cytokine stimulation had significant group effects for increasing MMP-1 (p=0.007) and MMP-3 (p<0.001) expression. Compared to osteoarthritic cells, normal meniscus cells exhibited a greater mean increase in MMP-1 and MMP-3.
expression in response to cytokine stimulation (p=0.007, p=0.012). Normal meniscus cells also demonstrated a greater mean increase of MMP-1 expression at six hours than osteoarthritic meniscus cells (p=0.049), but by 24 hours this difference was not significant (p=0.608). Within the cytokine group, IL-1β and IL-6 significantly increased MMP-1 expression (respectively p=0.007 and p=0.009). Normal meniscus cells again exhibited a greater increase in MMP-3 expression at six hours (p=0.001) than osteoarthritic meniscus cells but was similar by 24 hours (p=0.096). Within the cytokine group, IL-1β and IL-6 significantly increased MMP-3 expression (respectively p<0.001 and p=0.013).

Immunoblot analysis of osteoarthritis meniscus cell MMP production demonstrated significant responses to cytokine stimulation. Densitometry measurements demonstrated significant MMP-1 increases of 1.43±0.29, 1.65±0.26, and 1.40±0.26 for IL-1β, IL-6, and TGF-α stimulation, respectively (p=0.043, n≥4 unique donors). MMP-3 increased significantly with 2.44 ± 0.84 change for IL-6, and increases of 1.38±0.30 for IL-1β and 1.64±0.36 for TGF-α (p=0.027, n≥5 unique donors). Subgroup analysis identified IL-6 as a more potent stimulus for MMP-1 and MMP-3 (p<0.05). MMP-8 production responded to cytokine stimulation but was more variable (p=0.108) than MMP-1 and -3. All osteoarthritic menisci produced some MMPs without stimulation, but some severely osteoarthritic meniscus cultures were unable to be further stimulated to increase MMP production (grade 4; data not shown). Meniscus cultures with high basal MMP production and undetectable increases in MMP production were excluded from the densitometry analysis. In comparison to normal meniscus cell MMP production, normal menisci increased their MMP-1 production in response to cytokine stimulation more than
ostearthritic menisci (p=0.003), but MMP-3 production did not reach statistical significance (p=0.068). Unlike normal menisci, cytokine stimulation did not increase MMP-13 production in osteoarthritic meniscus cells.

Osteoarthritic meniscus cells were also compared to osteoarthritic chondrocytes obtained from the same donor to determine if the two cell types differed in their response to cytokine stimulation. As shown in the MMP protein arrays (Figure 3A), human osteoarthritic meniscus cultures responded to cytokine stimulation with increased production of MMP-1,-3 and -8. Osteoarthritic chondrocytes demonstrated a different MMP profile with greater MMP-13 production (Figure 3A). The array results were confirmed with immunoblots, which demonstrated that osteoarthritic menisci responded to IL-1β, IL-6 and TGF-α with increased MMP-1 and -3 production (Figure 3B). While both osteoarthritic chondrocytes and menisci produced MMP-1 and -3, chondrocytes expressed more MMP-13 and ADAMTS-5 than osteoarthritic meniscus cells (Figure 3B).

**NF-κB pathway associated expression in normal meniscus cells**

Since FnF increased the many genes in the extracellular-matrix array (Table 1) and we previously demonstrated that FnF stimulated NF-κB pathway genes in chondrocytes 21, we selected FnF stimulation to evaluate the NF-κB family in meniscal cells. Twenty-six genes out of 84 on the NF-κB family array were significantly increased by FnF and only one, AGT, was decreased (Table 2). FnF stimulation increased expression of NF-κB components (NFκB1, NFκB1A, and Rel) and many target genes, including cytokines (IL-1α and -1β, IL-6, and IL-8) and chemokines (CSF1, CXCL1, and CXCL2). FnF additionally increased the expression of both receptors and ligands in the TNF-α family (CD40, Fas, LTB, TNFSF10 and TRAF2) as well as CD80 and CD83.
Treatment with FnF in the presence of the NFκB inhibitor hypoestoxide significantly altered the expression of a number of genes. The chemokines C4A and CCL2 were decreased in addition to the transcription factors STAT3 and EGR2. FnF with hypoestoxide decreased expression of the enzymes MAP2K6, NQO1, NR4A2, and PLA2. The receptor expression for IL1R2 was decreased while IL2RA was increased. Additional gene alterations that were not statistically significant may be found in Table S3.

Since FnF increased cytokine and chemokine gene expression in the NF-κB arrays, we used a protein array and tested conditioned media from FnF and cytokine treated cells to examine meniscus cytokine and chemokine production. Two different donors and exposures are shown to highlight the differences (Figure 4). All three pro-inflammatory stimuli increased production of CXCL1, CXCL2, CXCL3 (identified by the GRO antibody), CXCL5, CCL8 (MCP-2), CCL7 (MCP-3), GM-CSF, and MIP-3α. FnF and IL-1β increased IL-6 and CCL2 production. FnF and IL-6 increased IL-1β, and MIP-1β. FnF increased IL-1α while IL-1β uniquely increased MIF, and finally IL-6 increased IL-7. Since the arrays contained antibodies to detect IL-1β and IL-6, it is unclear if they increased their respective production or the blots were detecting the cytokines added to stimulate the cells.

To further examine FnF stimulation of the NF-κB pathway, we assessed p65 phosphorylation following stimulation by FN-F as well as IL-1β+IL-6. Phosphorylation of p65 increased following treatment with the pro-inflammatory factors and the addition of the NF-κB inhibitor hypoestoxide reduced p65 phosphorylation following stimulation with FnF (Figure 5). The overall level of phosphorylated p65 was statistically significant
Increased Catabolism in Meniscus Cells

Stone

(p=0.007), but hypoestoxide’s effect on IL-1β in combination with IL-6 stimulation was more variable. Additional cell cultures were also harvested for RNA and conditioned media analysis after cytokine stimulation and hypoestoxide inhibition. Stimulation treatment significantly altered MMP-1 (p<0.001) and MMP-3 (p=0.001) expression (Figure 6). Within the group, treatment with FnF or IL-1β+IL-6 significantly increased expression (MMP-1 p<0.01 for both hypoestoxide groups; MMP-3 p<0.05 for both hypoestoxide groups), while mean change following treatment in combination with hypoestoxide did not significantly differ from unstimulated control. The same trend was identified for MMP-1 and MMP-3 production.

Discussion

The clinical importance of the meniscus in osteoarthritis development is well documented 1-6; however, meniscus pathology in osteoarthritis is largely attributed to mechanically mediated loss of structural integrity 5, 12, 17, 25, 26. These biomechanical stress factors may lead to “osteoarthritis in the meniscus” which is proposed to be responsible for meniscus MRI changes observed during the early osteoarthritis development 27. Recent evidence suggests that the meniscus may have a more biologically active role in the complicated whole joint pathology of osteoarthritis 11, 15, 18, 28, 29. Many of these studies use animal meniscus specimens and are limited in their translation to human osteoarthritis pathogenesis 30. Our data support and expand upon previous gene expression reports 10,11,18 using normal and osteoarthritic human meniscus and further proposes a role for meniscus involvement in osteoarthritis pathogenesis.

The first objective was to identify extracellular matrix and MMP expression patterns in normal meniscus following pro-inflammatory stimulation. Early aberrations in
cytokine signaling are believed to be responsible for propagating the reactive and degradative responses in joint tissues that ultimately lead to osteoarthritis. Normal meniscus cells stimulated with pro-inflammatory factors vigorously increased their catabolic factor expression and protein production. The pattern of normal meniscus cell MMP production was consistent with that of osteoarthritic meniscus cells, although the diseased cells were less dynamic in their response. Normal meniscus cells were highly responsive to FnF, IL-1β and IL-6, while osteoarthritic menisci were more responsive to IL-6 and then IL-1β at the concentrations tested. Normal meniscus cells also responded earlier to stimulation than osteoarthritic meniscus cells, as evidenced by significantly greater increases in MMP expression at six hours than the osteoarthritic cells. This difference could be related to alterations in receptor density or inflammatory pathways in osteoarthritic cells. Meniscus cells produced a complementary pattern of MMP production to osteoarthritic chondrocytes in response to pro-inflammatory stimulation.

Alterations of MMP expression are important in osteoarthritis development and progression. MMP-1 degrades collagen type I which is the primary constituent of meniscal extracellular matrix. Increased MMP-1 activity may damage the structural integrity of the meniscus. MMP-3 (stromelysin-1) production is similarly important because it is upregulated in articular cartilage in early osteoarthritis. MMP-3 cleaves multiple matrix proteins and activates other MMPs, including MMP-1. The disease processes we observed through up-regulation and production of MMPs are likely present in the intact meniscus. This conclusion is supported by studies demonstrating increased MMP-3 and aggrecanase production in immunohistochemical analysis of partial
meniscectomy specimens\textsuperscript{15}, increased MMP-1 activity, proteoglycan release and nitric oxide release following IL-1\(\beta\) treatment in healthy pig meniscus explants\textsuperscript{34}, and increased expression of ADAMTS and MMPs in ovine meniscus following cytokine stimulation\textsuperscript{18}. Additional catabolic changes were identified with extracellular matrix analysis. A more dynamic gene response for MMP-8 was identified in normal meniscus cells, along with MMP-10. MMP-10 was reported in the fibrocartilaginous nucleus pulposus and was associated with increased gross and histological degeneration, pain, and increased IL-1 and substance P\textsuperscript{35}.

Pro-inflammatory stimulation also increased MMP-13 gene expression and production in normal meniscus cells. Our findings are consistent with increased MMP-13 following IL-1\(\alpha\) treatment in normal inner meniscus and articular chondrocytes\textsuperscript{18}. Increased MMP-13 gene expression in stimulated normal meniscus cells is also congruent with reported MMP-13 expression in partial meniscectomy specimens\textsuperscript{11}, and the inner region of the meniscus would be expected to constitute the majority of cells in partial meniscectomy. The meniscus cell phenotype is reported to become increasingly chondrocytic in the inner zones of the meniscus\textsuperscript{18,33,36}. The inner section is likely the first section to deteriorate in osteoarthritis progression and may explain in part why we did not see significant increases in MMP-13 production in our osteoarthritic meniscus cells.

Pro-inflammatory factors also altered expression of cell adhesion proteins. Alterations in the meniscus integrin receptor expression would be expected to alter cell-matrix interactions as previously shown for chondrocytes\textsuperscript{37} and is implicated in osteoarthritis pathogenesis\textsuperscript{10}. Cell adhesion markers VCAM-1, ICAM-1 and E-selectin
were also increased and were previously demonstrated to be present in hypertrophic and early osteoarthritic synovium and is involved in inflammatory cell recruitment to the synovium. ICAM-1 was specifically identified as increased in early osteoarthritis, while VCAM-1 was shown to be predictive of joint replacement for severe arthritis. Pharmacologic reductions of these molecules for early to mid-stage osteoarthritis of the knee was associated with improvements of pain and function.

Lymphotoxin β and GM-CSF were both increased and although they are primarily linked to rheumatoid arthritis, they are also reported in the osteoarthritic synovium. Future investigation may link these cytokines to the more fibroblastic cell phenotype in the meniscus or inflammatory cell recruitment. Catabolic expression was accompanied by a notable decrease in expression of the anabolic factor CTGF. CTGF was recently indentified in a rabbit model for promoting collagen production and healing of meniscus defects. The combination of abnormal cell recruitment and decreased anabolic factors could easily compromise wound healing.

Meniscus cells can be stimulated to produce matrix degrading enzymes which can impact cartilage matrix, but the tissue interaction is likely part of a more dynamic signaling network. In addition to the catabolic factors above, meniscus responded to pro-inflammatory factors with increases in cytokine and chemokine expression and production in a manner similar to chondrocytes. Multiple interleukins, including IL-1β and IL-6 used in our stimulation experiments, were increased in both expression and production. IL-1β was recently reported to be increased in osteoarthritic synovial fluid that increased in level with the severity of the disease. Additionally, treatment of articular chondrocytes and meniscus explants with IL-1α and IL-1β increased cartilage
and meniscus catabolic activity through increased MMP activity and nitric oxide release\textsuperscript{45}. Chemokines CXCL1, CXCL2, CXCL3, CCL8 (MCP-2), CCL7 (MCP-3), CXCL6 (GCP-2) were increased and may contribute to the development of inappropriate inflammatory cycles after injury\textsuperscript{9,28}. Our results support recently reported findings in an analysis of gene expression in meniscus tears, which found increased expression of IL-1\(\beta\), ADAMTS-5, MMP-1, MMP-9, MMP-13, and NFkB2 in patients with meniscus tears younger than 40\textsuperscript{11}. Cytokine and chemokine expression (including IL-1\(\beta\), TNF-\(\alpha\), MMP-13, CCL3, and CCL3L1) were greater in patients with a meniscus tear and concomitant ACL tear which indicates a more severe injury\textsuperscript{11}. Furthermore, we identified a more expansive list of cytokine and chemokine alterations and proposed that these alterations are at least in part mediated by the NF-\(\kappa\)B pathway.

The NF-\(\kappa\)B pathway is well studied in osteoarthritic chondrocytes. FnF stimulation of NF-\(\kappa\)B increases chondrocyte cytokine and chemokine production\textsuperscript{9,22,28,46}. In meniscus cells, FnF and cytokine directed p65 phosphorylation suggests that the NF-\(\kappa\)B pathway may be responsible for increased cytokine and chemokine production. Injured meniscus previously demonstrated elevated NF-\(\kappa\)B phosphorylation identified by immunohistochemistry\textsuperscript{16}.

Increased production of inflammatory factors may act in both autocrine and paracrine fashion, but these may also act on surrounding tissues through the synovial fluid. This mechanism for joint destruction is supported by a number of studies identifying these factors as increased in the disease state and detailing their deleterious effects on cartilage, bone and the synovium\textsuperscript{9,28,32}, which would likely suppress reparative cell functions and propagate a loss of matrix integrity. Additionally, these findings may better explain the
higher failures in meniscus repair in older patients \(^4,11,47\). Older patients with a previous
meniscus injury are likely producing increased matrix degrading enzymes as a function of
both the initial injury and age, and both factors are likely to contribute to disease
progression.

Our study carries common limitations of laboratory models. Primary cell culture was
the most efficient and precise model to analyze both protein and RNA responses to
stimulation; however, our findings should be interpreted with the understanding that cell
cultures may not directly mimic \textit{in vivo} cell behavior. This study sought to identify cell
alterations in normal meniscus tissue that may lead to the development of osteoarthritis.
Future studies may further explore the NF-\kappa B mechanistic pathway including MAP
kinases and disease progression in an animal model, which was beyond the scope of this
manuscript. Another limitation of the study is the inherent variability in the state of the
meniscus disease at the time of specimen acquisition. TKAs are most frequently
performed for the indication of pain and functional limitation from osteoarthritis, but the
indication encompasses a range of tissue destruction ranging from moderate to severe
cartilage eburnation and meniscus degradation. The larger standard deviation in MMP
expression and production in osteoarthritic tissue may be partially attributable to the
varied disease state. We opted to examine the entire cell population in the meniscus to
elucidate differences between the normal meniscus and the osteoarthritis disease state.
Additional studies have examined the differences in meniscus cell type \(^18,30\), so we
believe our characterization of normal and osteoarthritis human meniscus may add to a
better understanding of osteoarthritis pathogenesis following meniscal injury.
The role of the meniscus in osteoarthritis likely extends beyond the mechanical compromise of the meniscus structure to encompass biologic interactions. Meniscus secretion of inflammatory factors and matrix-degrading enzymes likely contributes to the development of pathology. While the full cell mechanism was not characterized, we believe that the increased expression of MMPs, cytokines, and chemokines in response to pro-inflammatory factors contributes to osteoarthritis pathogenesis in the meniscus and articular cartilage. The ultimate goal of this research is to identify early pathology in an effort to prevent, or at least attenuate, the development of osteoarthritis.

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**Author Contributions**

Stone: Conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding, collection and assembly of data.

Loeser: Conception and design, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding.
Long: Conception and design, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article.

Vanderman: Critical revision of the article for important intellectual content, final approval of the article, collection and assembly of data.

Clark: Critical revision of the article for important intellectual content, final approval of the article, collection and assembly of data.

Ferguson: Conception and design, analysis and interpretation of the data, critical revision of the article for important intellectual content, final approval of the article, obtaining of funding.

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Competing interests:
The authors have no competing interests to report. Funding sources disclosed above.

References


Figure 1: Response of normal meniscus cells to pro-inflammatory stimulation.

(A) MMP-1 and MMP-3 gene expression in meniscus cells. Primary cell cultures were stimulated with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml) or fibronectin fragments (FnF, 1 μM) and cells were harvested 24 hours after stimulation (n≥4 unique donors) [MMP1: *p=0.017 (IL-1β), p=0.021 (IL-6), ***p<0.001; MMP3: **p=0.003, ***p<0.001]. All real-time PCR data was normalized to internal control (unstimulated) for accurate full change comparisons. Error bars represent 95% confidence intervals. (B) Normal meniscus primary cultures stimulated with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml), or FnF (1 μM) (n=4 unique donors). Conditioned media was collected at 24 hours after stimulation and immunoblotted for MMP-1, -3, or -13. MMP-2 levels did not change and served as an additional loading control. Graphs to the right of the
immunoblots represent densitometric analysis of the immunoblots (line represents median, box 25% - 75% quartiles, and whiskers the 95% confidence interval).

**Figure 2: Changes in osteoarthritic meniscus gene expression following cytokine stimulation.**

Osteoarthritic meniscus primary cell cultures were stimulated with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml) (n≥4 unique donors) [mean increase in MMP-1 (p=0.060); MMP-3 (p=0.013)]. Cells were harvested 24 hours after stimulation. All real-time PCR data normalized to internal control (unstimulated cells) for accurate fold change comparisons. Error bars represent 95% confidence intervals.
Figure 3: Comparison of osteoarthritic meniscus and cartilage cells in response to cytokine stimulation. (A) Antibody MMP Array with conditioned media from osteoarthritic meniscal cells and chondrocytes following 24 hour stimulation with IL-1β (10 ng/ml) or IL-6 (10 ng/ml plus 25 ng/ml sIL6R). All protein arrays were developed simultaneously to enable direct comparisons and each protein on the array is presented in duplicate. (n=1, + indicates positive control) (B) MMP-1, -3, -8, and -13 and ADAMTS-5 production in osteoarthritic chondrocytes and meniscus cells. Immunoblot analysis of conditioned media from unstimulated controls (C) versus with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml) stimulated cultures (n=4 matched donors).
Figure 4: Protein array of conditioned media from normal meniscus following pro-inflammatory stimulation. Conditioned media from normal meniscus cells 24 hours after stimulation with either Fn-F (1 μM), IL-1β (10 ng/ml) or IL-6 (10 ng/ml plus 25 ng/ml sIL6R). The first donor is shown with shorter (A) exposure and the second donor with a longer exposure (B) to detect less abundant cytokines.
Figure 5: Normal meniscus p65 phosphorylation in response to pro-inflammatory stimulation.

Cells were stimulated for thirty minutes with either FnF (1 μM) or IL-1β (10 ng/ml) and IL-6 (10 ng/ml plus 25 ng/ml sIL6R) with or without hypoestoxide (HE, 25 μM), and cell lysates prepared. Lysates were then probed for phosphorylated-p65 (active form). Immunoblots were then probed for total-p65 and followed by β-actin as the loading control (n=5 individual donors).

Densitometric analysis identified significant increases in p65 phosphorylation (p=0.007). The blots were stripped and re-probed for total-p65 and β-actin. Total-p65 was present in lanes with minimal phospho-p65.
Figure 6: Response of normal meniscus cells to pro-inflammatory factors with and without the NF-κB inhibitor hypoestoxide.

(A) Cells were harvested for RNA collection 24 hours after stimulation with either with IL-1β (10 ng/ml) and IL-6 (10 ng/ml plus 25 ng/ml sIL6R) or FnF (1 μM) with and without the inhibitor hypoestoxide (HE, 25 μM). All real-time PCR data normalized to internal control (unstimulated cells) for accurate fold change comparisons. MMP-1 and MMP-3 expression significantly changed with treatment groups [MMP-1 overall p<0.001, **p=0.01; MMP-3 overall p=0.001; *p=0.04].

Error bars represent 95% confidence intervals (N=5 independent donors). (B) The conditioned media was also probed for MMP production (n=5 independent donors).
Table 1: Quantitative Real Time PCR Array for Selected Extracellular Matrix Related Genes

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<tr>
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<th>IL-1β p Value</th>
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<td>ITGB1</td>
<td>Integulin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)</td>
<td>0.1927</td>
<td>1.36</td>
<td>0.0452</td>
<td>1.86</td>
<td>0.3471</td>
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<td>Integulin, beta 2 (complement component 3 receptor 3 and 4 subunit)</td>
<td>0.2652</td>
<td>4.34</td>
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<td>0.0196</td>
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<td>MMP1</td>
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Red highlights indicate p<0.05. Green highlights fold changes greater than +3 and blue highlights fold changes less than -3.
Table 2: NF-κB family gene targets.

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<th>Gene</th>
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<th>FnF</th>
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<td>IL1B</td>
<td>Interleukin 1, beta</td>
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<td>IL1RN</td>
<td>Interleukin 1 receptor antagonist</td>
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<td>IL6</td>
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<td>IL8</td>
<td>Interleukin 8</td>
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<td>CXCL10</td>
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<td>CD40</td>
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<td>CD80 molecule</td>
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<td>CD83</td>
<td>CD83 molecule</td>
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<td>LTB</td>
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<td>NFKB2</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)</td>
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<tr>
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<td>ICAM1</td>
<td>Intercellular adhesion molecule 1</td>
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</table>
**Supplementary Material**

Table S1: Grading system used for cartilage and meniscus specimens.

<table>
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<tr>
<th>Grade</th>
<th>ICRS Macroscopic Cartilage Grade</th>
<th>Meniscus Adaptation</th>
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<tbody>
<tr>
<td>Grade 0</td>
<td>Normal cartilage</td>
<td>Normal meniscus</td>
</tr>
<tr>
<td>Grade 1</td>
<td>Superficial lesions</td>
<td>Near-normal with minor fibrillations</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Lesions &lt;50% of cartilage thickness</td>
<td>Small, scattered cracks or fissures, some fibrillation</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Lesions &gt;50%</td>
<td>Small tear, extensive fibrillations</td>
</tr>
<tr>
<td>Grade 4</td>
<td>Abnormal cartilage with exposed subchondral bone</td>
<td>Large tear, extensive fibrillations</td>
</tr>
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Table S2: Quantitative Real Time PCR Array for Extracellular Matrix Related Genes

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<tr>
<th>Gene</th>
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<th>FnF p Value</th>
<th>FnF Fold Change</th>
<th>IL-1β p Value</th>
<th>IL-1β Fold Change</th>
<th>IL-6 p Value</th>
<th>IL-6 Fold Change</th>
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<tr>
<td>ADAMTS1</td>
<td>ADAM metallopeptidase with thrombospondin type 1 motif, 1</td>
<td>0.7073</td>
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<td>0.7195</td>
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<td>0.9486</td>
<td>-1.14</td>
<td>0.8934</td>
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<td>CD44</td>
<td>CD44 molecule (Indian blood group)</td>
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<td>0.1609</td>
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<td>Contactin 1</td>
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<td>ITGB1</td>
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<td>0.1927</td>
<td>1.36</td>
<td>0.0452</td>
<td>4.79</td>
<td>0.3471</td>
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<tr>
<td>ITGB2</td>
<td>Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)</td>
<td>0.2652</td>
<td>4.34</td>
<td>0.0839</td>
<td>5.92</td>
<td>0.0196</td>
<td>7.27</td>
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<td>ITGB3</td>
<td>Integrin, beta 3 (platelet glycoprotein IIla, antigen CD61)</td>
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Red highlights indicate p<0.05. Green highlights fold changes greater than +3 and blue highlights fold changes less than -3.
### Table S3: Quantitative Real Time PCR Array for NF-κB Family Genes and Targets

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<td>0.8250</td>
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<td>Interleukin 1 receptor antagonist</td>
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<td>IL6</td>
<td>Interleukin 6 (interferon, beta 2)</td>
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<td>LTA</td>
<td>Lymphotoxin alpha (TNF superfamily, member 1)</td>
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<td>Mitogen-activated protein kinase kinase 6</td>
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<td>MITF</td>
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<td>MMP9</td>
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<td>0.8604</td>
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<td>PLAU</td>
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<td>1.97</td>
<td>0.0380</td>
<td>-9.97</td>
<td>0.0115</td>
<td>-19.59</td>
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<td>Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)</td>
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<td>14.21</td>
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<td>3.13</td>
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<td>V-rel reticuloendotheliosis viral oncogene homolog B</td>
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<td>0.4788</td>
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<td>0.8646</td>
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<td>0.0621</td>
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<td>XIAP</td>
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Red highlights indicate p<0.05. Green highlights fold changes greater than +3 and blue highlights fold changes less than -3.
Pro-inflammatory stimulation effects on the Hypoxia-inducible factor (HIF) pathway in normal and osteoarthritic meniscus and the effects of Epas1 insufficiency on joint protection in mice.

Austin V. Stone MD, Richard F. Loeser MD, Michael F. Callahan PhD, Margaret A. McNulty PhD, David L. Long, Raghunatha R. Yammani PhD, Kadie Vanderman MS, Stephanie Clark, Cristin M. Ferguson MD
Abstract

Objective: Meniscus injury and the hypoxia-inducible factor (HIF) pathway are independently linked to osteoarthritis pathogenesis, but the HIF pathway has not been explored in the meniscus. We sought to identify and evaluate the response of the HIF pathway in normal and osteoarthritic meniscus and to examine the effects of Epas1 (HIF-2α) insufficiency in mice on early osteoarthritis development.

Methods: Normal and osteoarthritic human meniscus specimens were obtained and used for immunohistochemical evaluation and cell culture studies for the HIF pathway. Meniscus cells were treated with proinflammatory stimulation, including interleukins (IL)-1β, IL-6, transforming growth factor (TGF)-α and fibronectin fragments (FnF). Transcription target genes were also evaluated with HIF-1α and HIF-2α (Epas1) overexpression and knockdown. Wild-type (n=36) and Epas1+/− (n=30) heterozygous mice underwent the destabilized medial meniscus (DMM) surgery and were evaluated at two and four weeks post-operatively for osteoarthritis development using histology.

Results: HIF immunostaining and HIF gene expression did not differ between normal and osteoarthritic meniscus. While pro-inflammatory stimulation significantly increased both catabolic and anabolic gene expression in the meniscus, HIF-1α and Epas1 expression levels were not significantly altered. Epas1 overexpression significantly increased Col2a1 expression. Both wild-type and Epas1+/− mice developed osteoarthritis following DMM surgery. There were no significant differences between genotypes at either time point.

Conclusion: The HIF pathway is likely not responsible for osteoarthritic changes in the human meniscus. Additionally, Epas1 insufficiency does not protect against osteoarthritis development in the mouse at four weeks after DMM surgery. The HIF pathway may be more important for protection against catabolic stress.
Introduction

The hypoxia induced factor (HIF) pathway is implicated in osteoarthritis pathogenesis. HIF-1α is a transcription factor that serves many anabolic functions by maintaining metabolic homeostasis through cell growth and protection, while HIF-2α is implicated in an array of anabolic and catabolic functions. HIFs are reportedly more active in low oxygen tension tissues, such as cartilage and meniscus, secondary to decreased degradation by prolyl hydrolyase domain-containing protein. HIF-1α is a critical regulator of glycolytic and oxidation pathways and helps to optimize ATP production and limit reactive oxygen species (ROS) accumulation in normoxia and hypoxia. HIF-2α (encoded by the gene Epas1) shares many metabolic targets with HIF-1α but is more cell type specific and is regulated differently. HIF-2α does not appear to be constitutively expressed in a manner similar to HIF-1α in developed tissues; however, in the developing mouse embryo it is critical in the development of multiple organ systems including the musculoskeletal system. Another component of the HIF pathway, HIF-3α, was recently reported to be involved in regulating HIF-1α and HIF-2α in mice; however, its role remains unclear since a previous report identified very low expression levels in mouse chondrocytes.

Both HIF-1α and HIF-2α have been identified in articular cartilage. In articular chondrocytes, HIF-1α is reported to be active in both normoxia and hypoxia and controls much of cartilage homeostasis and chondrocytic genes. The role of HIF-2α in chondrocytes is more controversial. HIF-2α has been linked to anti-catabolic processes in human chondrocytes, including increased expression of matrix-related genes, Sox9, Col2a1 and Col91a, along with Mig6, inhibin A (InhbA), cartilage matrix protein chondromodulin-1 (Chm-1), and cartilage growth factor Gdf10. HIF-2α was also linked to the development of osteoarthritis in human, rabbit, and mouse chondrocytes. Human chondrocytes displayed increased HIF-2α immunostaining and Epas1 expression in damaged cartilage. In rabbit and mouse
articular chondrocytes, Epas1 was identified to regulate multiple matrix metalloproteinases (MMPs), IL-6 production and chondrocyte apoptosis. Heterozygous deletion of Epas1 in Epas1+/- mice provided resistance to surgically induced osteoarthritic changes using the destabilized medial meniscus (DMM) model. The mechanism for resistance was not explored and remains unclear. Transgenic mice over-expressing Epas1 had increased osteoarthritic changes in the knee cartilage. Levels of HIF-2α were additionally increased in osteoarthritic cartilage of the DMM mice. Similar trends were seen in human osteoarthritic cartilage: levels of HIF-2α and its targets were present in damaged human osteoarthritic cartilage, while levels were lower in normal and undamaged osteoarthritic cartilage compared to damaged regions of the osteoarthritic cartilage. The role of HIF-1α was not explored in the mouse and osteoarthritis studies.

Osteoarthritis is an enigma in joint disease, but it is increasingly apparent that the disease is a function of whole joint pathology and not solely the articular cartilage. A growing body of evidence supports the link between meniscus injury and osteoarthritis. The HIF pathway in the meniscus may provide an opportunity to better understand meniscal osteoarthritis pathogenesis. HIF-1α is also reported in meniscus cells with greater expression in the more hypoxic inner regions than the vascularized outer regions of the meniscus. Expression levels varied between meniscus regions, so it is believed that HIF-1α helps to direct meniscus cell phenotype and potentially the repair process through differentially regulated collagen expression in inner and outer meniscus cells. Meniscus cell HIF-1α targets may differ from HIF-1α targets in chondrocytes, which may contribute to the differences in cell phenotype. To date, no study investigated the role of HIF-2α expression in the meniscus.

The purpose of this study was to identify both HIF-1α and HIF-2α in normal and osteoarthritic meniscus cells, and then to identify a potential role for the HIF pathway in the osteoarthritis pathogenesis. Pro-inflammatory stimulation implicated in osteoarthritis...
pathogenesis was used to determine meniscus responses in context of the HIF pathway. A second goal of this research is to study the meniscus in the context of whole joint pathology in surgically induced osteoarthritis in wild-type and Epas1 knockdown mice. By identifying a potential role for the HIF pathway in osteoarthritis, it may be possible to modify the HIF pathway in multiple joint tissues to modify osteoarthritis pathogenesis.

**Methods**

*Knee Tissue Acquisition*—Our institutional review board and animal care and use committee approved these protocols. Normal human meniscus specimens (n=16, age range 25-58 years) were procured through the National Disease Research Interchange (NDRI, Philadelphia, PA) or the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) while osteoarthritic menisci were obtained from patients undergoing total knee arthroplasty (TKA) for osteoarthritis (n=31, age range 44-83 years). Synovial tissue was removed. Meniscus tissue was macroscopically graded according to a modified International Cartilage Research Society Cartilage Morphology Score (Table S1).

*Human meniscus immunohistochemistry*—Human meniscus samples were fixed in 4% paraformaldehyde and processed for paraffin embedding. Radial sections (4 μm) were obtained and placed on slides for histologic evaluation. Samples were then deparaffinized and stained for immunohistochemistry or immunofluorescent evaluation. Meniscus sections were incubated overnight with either anti-HIF-1α (R&D Systems) or anti-HIF-2α (R&D systems). For diaminobenzidine (DAB) development, the appropriate biotinylated secondary antibody was used and developed according to the manufacturer’s protocol (DAB Peroxidase Substrate Kit, #SK-4100, Vector Laboratories). Immunofluorescence staining used Texas Red secondary antibodies (Alexafluor488, Molecular Probes) and nuclei were counterstained with 4’6-diamidino-2-phenylidole (DAPI, Vectashield #H-1200, Vector Laboratories). Slides were imaged using either light microscopy or fluorescent imaging with the appropriate filters.
**Cell Culture and Stimulation:** Normal and osteoarthritic human meniscus and articular chondrocytes were as previously described\textsuperscript{33}. Prior to stimulation, primary cultures were incubated overnight in serum-free media (DMEM/F12) and then treated for either 6 or 24 hours with one of the following: 10 ng/ml IL-1β; 10 ng/ml IL-6 with 25 ng/ml soluble IL-6 receptor; TGF-α 20 ng/ml (all from R and D Systems); hypoxestoxide (25 μM); or fibronectin fragments (FnF), a recombinant fragment of fibronectin protein containing domains 7-10 of full length fibronectin (at 1 μM; gift from Harold Erickson, Duke University). For viral and small-interfering RNA experiments, cells were plated and allowed to acclimate for 48 hours prior to infection.

The Epas1 virus was generated by Vector BioLabs (Philadelphia, PA) using a cDNA plasmid for *Homo sapiens* endothelial PAS domain protein 1 (GenBank ID: BC051338.1). The adenoviral shuttle vector contained a GFP coexpression reporter for confirmation of infection and expression in cell culture. The virus was serial amplified in HEK293 cells then was harvested and titrated. The custom construct was used in cell culture studies. The HIF-1α virus (ADV-100, Cell Biolabs, Inc.) also contained a GFP reporter and was expanded in culture using HEK293 cells and was titred using QuickTiter™ Adenovirus Quantitation Kit (VPK-106, Cell Biolabs, Inc).

For the viral infection, $4 \times 10^9$ viral particles in 1 mL serum free DMEM/F-12 media (Gibco) plus 25 μL of 1M CaCl$_2$ (Sigma) were used per $10^6$ cells with control samples receiving an empty vector. Cells were incubated for 2 hours at 37°C then washed twice in 10% media. Fresh 10% media was added to the cells which were allowed to incubate for 2 days. For the siRNA mediated knockdown, cells were rinsed twice with PBS and changed to 1 mL 10% media without antibiotics for 24 hours at 70% confluence. Cells were transfected with Lipofectamine 2000 (#11668, Invitrogen) according to the manufacture’s protocol. Briefly, 100 pmol of siRNA for non-specific control, HIF-1α or Epas1 (Cell BioLabs), and 5 μL lipofectamine 2000 were diluted
separately in 250 μL Opti-MEM + glutamax 1x reduced serum media 1x (Invitrogen). 250 μL of each diluted siRNA and lipofectamine was incubated together for twenty minutes, and each well of cells in a six-well plate received 500 μL transfection reagent and siRNA added to existing media and was allowed to incubate for 24 hours. Protein lysates of transfected cells were obtained using the Pierce NE-PRE Nuclear and Cytoplasmic Extraction Reagents and following the manufacture’s protocol [78833 (Thermo Scientific)]. RNA was extracted from parallel experiments using the TRIzol method (Invitrogen) according to the manufacture’s protocol.

RNA was quantified (Nanodrop, ThermoScientific) and verified (BioAnalyzer Chip, Agilent) to ensure high quality RNA. The reverse-transcription PCR generated cDNA (RetroScript Kit, Ambion). Real-Time PCR was performed using the Applied Biosystems 7900HT thermocycler with TaqMan Universal PCR MasterMix and Taqman Gene Assay (Life Technologies: HIF-1α Hs00153153_m1; epas1 Hs01026149_m1, mmp1 Hs00899658_m1; mmp3 Hs00968305_m1; mmp13 Hs00233992_m1; col1a1 Hs00164004_m1; col2a1 Hs00264051_m1; col3 Hs00943809_m1; sox9 Hs01001343_g1; acan Hs00153936_m1; VEGF Hs00900055 _m1; GAPDH Hs02758991_g1). Data was analyzed using the ΔΔCT method in Microsoft Excel (Microsoft).

**Immunoblot analysis** – For protein analysis, nuclear and cytoplasmic lysate samples containing equal amounts of total protein were loaded onto polyacrylamide gels, separated by SDS-PAGE, transferred to nitrocellulose (Odyssey, Invitrogen) and probed with the primary antibody [anti-Hif-1α (MAB1536, R&D Systems); anti-Hif-2α (ab20654, Abcam)] and appropriate secondary antibody (Cell Signaling). Blots were visualized with chemiluminescence (Amersham ECL, GE Life Sciences) as previously described or the SuperSignal West Femto Substrate system [#34095 (Thermo Scientific)]. To verify loading of equal amounts of protein, blots were stripped and re-probed with antibodies to Lamin B (#101-B7, Millipore) for nuclear
fractions or LDH (#20-LG22, Fitzgerald) for cytoplasmic fractions with the appropriate secondary antibodies (Cell Signaling).

Mouse Destabilized Medial Meniscus Model – C57/BL6129S7 mice (10-12 weeks old) were acquired from the Jackson laboratories. All controls were wild type mice littermates since stock order C57/B6129SF2 mice provide only an approximate control. Each mouse was genotyped for wild type (WT; Epas1^+/+) or Epas1 heterozygote (Epas1^+/−) using the primers for allele Epas1^tm1Rus [mutant oMR0092 (5′—AAT CCA TCT TGT TCA ATG GCC GAT C—3′); wild-type oMR6168 (5′ —CGT GCT CTG GAG AGG TTA GG—3′); common oMR6169 (5′ —GGC ATG AAG ATG TGA GTT CG—3′); Figure S1]. The heterozygote was chosen because the homozygous deletion is embryonic lethal10. Mice (WT=36; Epas^+/−= 30) underwent destabilization of the medial meniscus (DMM) surgery. Mice were anesthetized under isoflurane in the knee prepared for aseptic surgery. The knee joint was opened under direct microscopic visualization with a minimally invasive parapatellar incision, and then the medial meniscus and the meniscotibial ligament identified. The medial meniscotibial ligament was transected and the skin and joint closed. Animals were monitored postoperatively and treated with the appropriate analgesia. Mice were allowed unrestrained movement throughout the post-operative period. Mice were euthanized at post-operative week two (WT=17, Epas^+/−=13) and four (WT=19, Epas^+/−=17) and knee joints were harvested and prepared for histology according to the methods previously described34,35.

Statistical Analysis – All gene expression experiments and mouse data were analyzed using multivariate general linear models with appropriate post-hoc testing when indicated (Tukey and Holm-Sidak). Correlations were determined using Spearman’s Rho. Statistical calculations were performed in SPSS (IBM) and graphs generated in Prism v5.02 (GraphPad Software, Inc.). For all analyses, statistical significance was set at p≤0.05.
Results

**HIF expression in normal and osteoarthritic meniscus cells**

To evaluate the difference between HIF expression in normal and osteoarthritic meniscus cells, we first identified the presence of HIF-1α and HIF-2α in normal and osteoarthritic menisci by immunohistochemistry. Both HIF proteins were identified in normal and osteoarthritic tissue and HIF-1α and HIF-2α localized to the nucleus (Figure 1, S2). The percentage of positively stained cells was not different (Figure 1): HIF-1α: normal (55.3% ± 2.6%, n=2), osteoarthritic (75.3% ± 11.7%, n=5); HIF-2α: normal (75.4% ± 10.3%, n=2), osteoarthritic (83.9% ± 13.7%, n=5). Results reported are mean ± standard deviation.

We then examined basal expression of HIF-1α and HIF-2α (Epas1) genes in primary meniscus cell culture. The unstimulated control groups from both the six and 24 hour time points in our pro-inflammatory stimulation studies were compared. No significant differences were observed in basal HIF family expression between normal and osteoarthritic menisci (Figure 2, main effects: group p = 0.409; time p = 0.285). HIF-1α expression did not differ between normal and osteoarthritic meniscus cells (Figure 1A, p = 0.965); likewise, the relative expression of HIF-1α and Epas1 was not significantly different between normal and osteoarthritic meniscus cells (Figure 2B, p = 0.522). To confirm that ratio of HIF-1α to Epas1 expression did not differ between osteoarthritic and normal meniscus, the relative expression of HIF-1α was divided by that Epas1 for each point and plotted (Figure 2C). There was no significant group or time effect (group p= 0.669; time p = 0.331). Since basal expression and the expression ratio did not differ between the normal and osteoarthritic menisci in culture, we continued to explore the role of pro-inflammatory stimulation on expression of HIF-1α and Epas1.

Pro-inflammatory mediators are known to be involved in osteoarthritis pathogenesis in chondrocytes. Primary meniscus cell cultures were stimulated with the pro-inflammatory...
mediators IL-1β, IL-6, and TGFα. HIF expression was assessed at six and 24 hours (Figure 3).

There were no significant effects of time (HIF-1α, p=0.244; Epas1, p=0.337) or stimulants (HIF-1α, p=0.434; Epas1, p=0.373) in osteoarthritic meniscus cells.

Gene expression in normal meniscus cells was also evaluated following pro-inflammatory stimulation. In the second set of experiments, fibronectin fragments (FnF) were also included since these were previously demonstrated to be pro-inflammatory in normal meniscus [see Chapter 3] and chondrocytes [37]. Fibronectin fragments are cartilage breakdown products released following chondral insult [37]. Gene expression in normal meniscus cells was evaluated at six and 24 hours following stimulation. Pro-inflammatory stimulation in normal meniscus cells did not significantly increase HIF expression (HIF-1α, p=0.434; Epas1, p=0.373). For clarity, since no difference existed between the six and 24 hour time points in HIF expression (HIF-1α, p=0.244; Epas1, p=0.337), only the 24-hour time point is shown (Figure 4A). HIF-1α and Epas1 gene expression in normal meniscus cell did not differ significantly from osteoarthritic meniscus cell response (HIF-1α, p=0.459; Epas1, p=0.433).

To further evaluate possible HIF pathway responses, we evaluated pro-inflammatory effects on selected catabolic and anabolic genes. The selected genes are both relevant to the meniscus and reported targets of HIF-1α or Epas1 or both. Pro-inflammatory stimulation significantly increased MMP1 expression with fold increases of: IL-1β, 21.6 ± 9.8 (p=0.002); IL-6, 17.5 ± 7.1 (p=0.005); and FnF, 24.8 ± 8.4 (p=0.001) [Figure 4B]. MMP3 expression was also increased: IL-1β, 14.4 ± 6.1 fold (p=0.003); IL-6, 7.46 ± 1.5 fold (p=0.139); and FnF, 20.0 ± 7.9 fold (p=0.001) [Figure 4B]. Fewer significant increases were identified in anabolic genes, but interestingly, TGFα increased collagen type II expression (5.65 ± 2.4 fold, p= 0.010) and FnF significantly increased Sox9 expression (10.3 ± 4.2 fold, p=0.0002; Figure 4C).
Meniscus HIF expression in response pro-inflammatory stimulation was then correlated to selected genes. Results are located in Table 1. HIF-1α expression positively correlated with Epas1 expression (R=0.662, p<0.001). HIF-1α and Epas1 expression positively correlated with both MMP1 (R=0.403, p>0.001; R=0.408, p<0.001) and MMP3 expression (R= 0.641, p<0.001; R= 0.526, p<0.001). Both HIF-1α and Epas1 positively correlated with Sox9 expression (R=0.515, p<0.001; R=0.700, p<0.001) and the Sox9 target, collagen type II (R=0.472, p=0.003; R=0.501, p=0.002). Sox9 was also correlated with collagen type II (R=0.491, p=0.002).

**HIF expression and the NF-κB pathway**

Many of the transcription targets previously linked to Epas1 in chondrocytes are also increased with NF-κB activation. Epas1 signaling activity was reported to be partially dependent on NF-κB activations. We examined HIF expression in normal and osteoarthritic meniscus cells treated with the NF-κB pathway inhibitor, hypoestoxide. Treatment with hypoestoxide did not significantly change HIF expression in normal cell cultures (Figure 5A), but by 24 hours HIF expression was significantly reduced in osteoarthritic meniscus cells (Figure 5A; p=0.023). The hypoestoxide treatment had a greater effect on HIF-1α than Epas1 expression (p=0.007). Reduced HIF expression by hypoestoxide treatment may be related to a tonic activation of the NF-κB pathway in osteoarthritic meniscus cells. Normal meniscus cells were then treated with pro-inflammatory stimuli known to increase NF-κB activity with and without the addition of hypoestoxide. FnF significantly increased HIF-1α and Epas1 (p=0.009) expression while the addition of hypoestoxide reduced expression to a level not significantly different from control (p=0.886). The differences between HIF-1α and Epas1 responses were not significant (p= 0.250).

**HIF overexpression and knockdown in normal meniscus cells**

The relationship between HIF-1α and Epas1 and their downstream transcription targets, was evaluated with HIF-1α or Epas1 gene overexpression and knockdown. Following gene overexpression or knockdown of HIF-1α or Epas1, nuclear and cytoplasmic fractions of cell
lysates were examined for the presence of the target proteins (Figure 6). The infection with either HIF-1α or Epas1 successfully increased target protein production with the presence of the protein product located in the nucleus where it is active. Inhibition of the target gene was respectively accomplished with siRNA; however, we were unable to detect HIF-1α or HIF-2α protein production in the nonspecific siRNA control. We consequently confirmed siRNA inhibition by analyzing HIF gene expression and confirmed successful reduction (Figure 7A). Gene expression was then used to analyze the relationship to target genes.

For overexpression or knockdown experiments, the target genes were expanded to include additional transcription targets of HIF-1α or Epas1. Adenoviral and siRNA treatment significantly altered the respective expression of HIF-1α (p=0.009) and Epas1 (p<0.001) (Figure 7). While the expression of MMPs were not significantly influenced by knockdown or adenoviral treatment [MMP1 (p= 0.327); MMP3 (p= 0.119); MMP13 (p= 0.207)], the expression of collagens type I and II were significantly altered (p= 0.014, p= 0.042) (Figure 7). Collagen type I expression was decreased by both HIF-1α (p=0.077) and Epas1 (p=0.044) overexpression, while collagen type II expression was increased by the Epas1 adenovirus. Collagen type III, aggrecan, and Sox9 were not significantly altered (p= 0.488, p= 0.414, p= 0.907).

The HIF transcription target genes were compared to HIF-1α and Epas1 for potential correlations (Table 2). Significant correlations include a negative correlation between HIF-1α and Epas1 (R=−0.609, p=0.012). Epas1 positively correlated with the known HIF target, VEGF (R=0.546, p=0.035). Both aggrecan (Acan) and collagen type II correlated with Sox9 (R=0.756, p=0.001; R=0.582, p=0.018). The remainder of the correlation coefficients and significance values are delineated in Table 2.

**Effect of Epas1 heterozygote deletion on the destabilized medial meniscus model of osteoarthritis in mice**
The role of HIF-2α in the development of osteoarthritis was explored using the established mouse destabilized medial meniscus (DMM) model. An Epas1 heterozygote knockout mouse was used to evaluate potential joint protective effects of Epas1 deletion. Mice were evaluated two and four weeks postoperatively after destabilization of the medial meniscus. Representative histologic images from the four week time point are shown in Figure 8A.

As evidenced by both the histology sections and the ACS scores, the DMM model was successful in producing osteoarthritic changes (Figure 8A, Table S1). Cartilage thinning, fissuring, and chondrocyte cell death with nuclear dropout were all evident in the medial compartment. The main effects were not significant for mouse genotype (p=0.687) or time point (p=0.245). Osteoarthritis severity was significantly different for the medial versus lateral compartments (p<0.001). This difference is expected with a surgical model that initiates osteoarthritis in the medial compartment. Compartment differences were significant for the following variables: ACS score (p<0.001), articular cartilage area (p<0.001), articular cartilage thickness (P = 0.001), subchondral bone area (p<0.001), subchondral bone thickness (p<0.001), chondrocyte cell density (p<0.001), chondrocyte number (p<0.001), and meniscus area (p<0.001). There were no differences between compartments in meniscus cell density (p= 0.413) or meniscus cell viability (p= 0.377). These differences were not significant between gene type or side. A complete list of means for all variables may be found in Table S2.

**Discussion**

The HIF pathway and its regulation in joint tissues is complex and poorly defined. The first objective of the study was to demonstrate the presence of HIF proteins in the meniscus. Previous literature is frequently focused on either HIF-1α or Epas1; we opted to examine HIF-1α and HIF-2α genes, since the effect of these two mediators are likely exerted in concert. HIF-1α and Epas1 gene expression was identified in both normal and osteoarthritic meniscus. Epas1 was
hypothesized to be upregulated in osteoarthritic meniscus tissue based on the recent reports suggesting its involvement in osteoarthritis pathogenesis in articular cartilage\textsuperscript{6, 15, 25, 26}. While similar expression of HIF-1\(\alpha\) and HIF-2\(\alpha\) in both normal and osteoarthritic meniscus tissue was contradictory to the reports in osteoarthritic human and mouse cartilage\textsuperscript{6, 25, 26}, our findings are congruent with reported HIF-1\(\alpha\) and HIF-2\(\alpha\) expression in normal and osteoarthritic articular chondrocytes\textsuperscript{21}. Since both HIF-1\(\alpha\) and HIF-2\(\alpha\) were previously demonstrated to be responsive to inflammatory stimulation\textsuperscript{6, 15, 21, 25-28, 38, 39}, we opted to explore HIF responses to pro-inflammatory stimulation in the meniscus.

Pro-inflammatory stimulation was expected to increase Epas1 to a greater degree than HIF-1\(\alpha\) expression. The cytokines IL-1\(\beta\) and IL-6 did not increase HIF-1\(\alpha\) or Epas1 expression in meniscus cells, but FnF increased relative Epas1 and HIF-1\(\alpha\) expression. Cytokine stimulation did not alter the expression level of Epas1 in meniscus cells as was previously described in chondrocytes\textsuperscript{6, 15, 29}. Yang et al. described Epas1 induction by multiple pro-inflammatory cytokines, including TNF-\(\alpha\), IL-1\(\beta\), IL-17 and IL-21, but did not place these results in the context of HIF-1\(\alpha\)\textsuperscript{6}. HIF-1\(\alpha\) is also demonstrated to be increased by pro-inflammatory stimulation\textsuperscript{21, 27, 28, 39}. While Yang et al., Ryu et al., and Siato et al. linked the increased expression of Epas1 to increasing catabolic activity\textsuperscript{6, 15, 25, 26}, many authors have identified catabolic stress induction of HIF-1\(\alpha\) as a protective and repair response in chondrocytes\textsuperscript{5, 5, 9, 21, 24, 27, 28}. In osteoarthritic chondrocytes catabolic stress, including IL-1\(\beta\), where it was responsible for inducing HIF-1\(\alpha\) expression and was upregulated in more damaged regions of cartilage obtained at total knee arthroplasty\textsuperscript{28}. Both HIF-1\(\alpha\) and HIF-2\(\alpha\) were demonstrated to control energy production and share many of the same targets which was theorized to improve tissue repair\textsuperscript{10, 11, 40}. Furthermore, HIF-1\(\alpha\) was predominantly attributed to control anabolic expression and of chondrocytes and meniscus cells\textsuperscript{19}. 


Downstream transcription target genes of the HIF pathway were further explored in response to pro-inflammatory stimulation. Following stimulation, HIF-1α expression positively correlated with Epas1, which may suggest a common pathway of induction or synergistic effects. Both HIF-1α and Epas1 also positively and significantly correlated with MMP1 and MMP3 expression. MMP1 and MMP3 were reported to be targets of Epas1, in addition to MMP9, MMP12 and MMP13, in osteoarthritic chondrocytes; however, the increase in stimulated meniscus cells may be attributable to a HIF-independent pathway. We previously established that MMP1 and MMP3 transcription and translation was increased by pro-inflammatory stimulation in normal and osteoarthritic meniscus cells [see Chapter 3]. In chondrocytes and meniscus cells, MMP increases are linked to multiple inflammatory pathways, including NF-κB. In the overexpression studies, neither MMP1 nor MMP3 correlated with Epas1 expression, and MMP actually exhibited a significant negative correlation with MMP3 expression. Negative correlation of HIF-1α with MMP3 parallels the reported down regulation of MMP13 by HIF-1α. The level of increased MMP expression following pro-inflammatory stimulation far exceeded the level of increase seen with Epas1 overexpression in normal meniscus. The lack of correlation in stimulation studies and the more profound increases in MMP expression with pro-inflammatory stimulation suggests that HIF-independent pathways may be responsible for enhanced MMP production.

The HIF relationship to many anabolic factors exhibited a more complex regulation than expected. HIF-1α and Epas1 expression in normal meniscus cells was positively correlated with both collagen type II expression and Sox9 expression following pro-inflammatory stimulation. Epas1 overexpression increased collagen type II expression. Sox9 was significantly correlated with collagen type II and aggrecan expression. Collagen type II is a major constituent of cartilage extracellular matrix, but is also reported in the inner zones of the meniscus. Sox9 is an important developmental gene found be responsible for the phenotypic development of both
meniscus and cartilage cells and upregulates chondrocytic matrix genes, including Col2a1, Col9a1, Col11a2 and aggrecan\textsuperscript{9,16}. The regulation of Sox9 in both meniscus cells and articular chondrocytes is unclear. HIF-1\(\alpha\) has been demonstrated to have a significant role in Sox9 regulation but much of this work was either completed in osteoarthritic cartilage and meniscus or in the mouse\textsuperscript{4,19,24}. More complete studies in human articular cartilage suggests that human tissue responds differently from that of mice\textsuperscript{8,9,27}. Sox9 induction was dependent on HiF-2\(\alpha\) and not on HIF-1\(\alpha\) in human articular chondrocytes\textsuperscript{16}. Increased HIF-2\(\alpha\) in human chondrocytes is also linked to increased Sox9 expression and increased expression of chondrocytic extracellular matrix genes, including collagen type II and aggrecan\textsuperscript{9,16}. These increases in cartilage specific genes were seen even in normoxia\textsuperscript{9}. HIF-2\(\alpha\) is also linked to the control of additional anti-catabolic responses in health human articular chondrocytes through upregulation of Sox9 independent genes, chondrocyte regulators Mig6 and inhibin A (InhbA), cartilage matrix protein chondromodulin-1 (Chm-1), and cartilage growth factor Gdf1\textsuperscript{17}. Mig6 is linked to tissue maintenance in chondrocytes, while inhibin A was reported to ultimately lead increased tissue inhibitor of matrix metalloproteinases (TIMP-1)\textsuperscript{17}. HIF-1\(\alpha\) was also linked to upregulation of inhibin A\textsuperscript{17}. Chondromodulin-1 was recently identified in the inner but not the outer meniscus and is suggested to prevent vascular ingrowth into the inner sections of the meniscus\textsuperscript{42}. These findings increasingly support the notion that the HIF activity may be induced by catabolic stress, but respond with an anabolic and anti-catabolic response.

The role for the NF-\(\kappa\)B pathway for regulating the HIF pathway remains poorly defined. We did observe a decrease in both HIF-1\(\alpha\) and Epas1 expression following NF-\(\kappa\)B inhibition in osteoarthritic meniscus. The decrease is consistent with previous descriptions of NF-\(\kappa\)B regulation, since both genes have been shown to have stabilized or upregulated at least in part through the NF-\(\kappa\)B pathway\textsuperscript{6,15,21,22}. While HIF-1\(\alpha\) and Epas1 do have interactions with the NF-\(\kappa\)B pathway, the decrease in HIF expression following NF-\(\kappa\)B inhibition may be the result of
inhibiting a larger cellular repair response in diseased or stressed tissue and less of sole consequence of NF-κB activation\textsuperscript{23, 27}.

The variability in HIF gene expression and the lack of clear correlations with reported targets is likely multifactorial. While HIF expression is reported to be influenced by cytokines\textsuperscript{6, 26, 38}, much of the regulation may occur at the protein level and not the expression level\textsuperscript{39}. Proteosome degradation of the HIFs is well described \textsuperscript{9, 23}, but its methods for activation and not just degradation appear complex\textsuperscript{40}. Additionally, HIF levels can be heavily influenced by prolyl hydroxylation as demonstrated in some studies \textsuperscript{8, 9, 27}, and may not be stabilized by inflammatory stimulation to the degree originally proposed in recent studies\textsuperscript{6, 15}. In many of the studies that examined only HIF-1α in cell culture, HIF-1α was stabilized with cobalt chloride which also enhances HIF-2α stability\textsuperscript{4, 20, 28}. Studies that examined HIF-1α in isolation using these stabilization methods were likely seeing some effect from HIF-2α. Specific sequences in the HIF genes play a role in protein binding and activation, so the sensitive balance between the two actually supports a delicate quantitative regulation more so than a simple qualitative relationship\textsuperscript{40}. The sensitivity in HIF regulation likely accounts for the reciprocal changes in HIF-1α and Epas1 expression following viral infection.

Another reason for increased variability observed in our studies may be related to the heterogenous composition of the meniscus. The meniscus is comprised of both chondrocyte and fibroblastic like cell types. If the primary role of the HIF family is to promote chondrocyte differentiation then responses of the HIF family within the individual cell type may be confounded given our mixed population of cells. The response of collagens to HIF-2α and induction of Sox9 expression may actually promote a more chondrocytic phenotype in meniscus cells. Collagen type I was previously shown to be down regulated by HIF-1α\textsuperscript{4}, but collagen type I is the major constituent of the meniscus extracellular matrix. Conflicting evidence also exists for the behavior of inner versus outer meniscus cells and fibroblasts\textsuperscript{19, 43, 44}. Additionally, HIF-1α has
been emphasized as important in maintaining the chondrocytic phenotype. The HIF family proteins may not be as important in osteoarthritis pathogenesis in meniscus cells, it appears that they have a very significant role in chondrocyte phenotype and differentiation. Future studies in human meniscus cells may place a greater emphasis on the role of the HIF pathway in development, maintenance and repair of inner versus outer meniscus cells.

The mouse model of osteoarthritis was used to examine the effect of Epas1 deletion on preventing osteoarthritic changes. In our mouse studies at two and four weeks, we did not see a significant difference between genotypes in joint protection. Our mouse model results were not consistent with the chondroprotective effects of Epas1 insufficiency reported by Yang et al., Ryu et al., or Siato et al. This finding may be attributed to multiple factors. The evidence of osteoarthritis by the four week time point also confirmed technical adequacy of the DMM surgery, and early osteoarthritic changes were both temporally and histologically congruent with previous reports. Our histologic grading system was more rigorous than that used by the previous Epas1 mouse studies and consequently identifies tissue specific and more subtle early disease alterations. Slides were also graded by a blinded expert (MAM) using the previously validated scoring system. It is possible that the chondroprotective effects of Epas1 insufficiency may become more evident at seven to eight weeks as previously reported. HIF-2α expression appears to target catabolic expression in some studies, but the relationship of HIF-1α was not explored in the recent studies in mice. Joint destruction in mice following local Epas1 transfection may be related to a complete distortion and overload of the HIF pathway, which is tightly regulated and highly sensitive. Reports in mice also linked HIF-2α role in regulating the pro-inflammatory cytokine IL-6 production and articular chondrocyte apoptosis. Epas1 may also not be as important in human osteoarthritic changes as evidenced by a large cohort study that refuted linking a functional single nucleotide polymorphism in the human Epas1 gene to osteoarthritis in a Japanese population. Although the objective of our mouse model
was not to examine all effects of Epas1 insufficiency, our results suggest that Epas1 insufficiency
does not significantly offer protection of the chondral surfaces nor influence meniscus
appearance.

The current study is limited by common limitations of laboratory models. Cell culture
responses may not directly mimic those of the intact meniscus in its native environment, but the
use of primary cell culture provided and effective modality for precise control of the stimulus and
measurement of gene expression and protein production. Cell culture studies were conducted in
normoxia, which does impact hypoxia sensitive genes. Previous reports suggested that HIF
proteins are stabilized by pro-inflammatory stimulation conditional experiments were it if
expression was still present and active under normoxic conditions6, 20, 21. Future studies may
explore the role of hypoxia on both HIF-1α and HIF-2α in the meniscus. Another limitation is
that it is possible to miss transcription alterations that may rapidly occur, although our time points
at six and 24 hours were chosen based on HIF increases in chondrocytes by six to 24 hours3, 4, 21.
We do not have protein product confirmation of many of the gene targets, but the HIF family
proteins are transcription factors and should be able to demonstrate their activity by increase in
target gene expression. As aforementioned, meniscus cells are heterogenous population in our
results may not be directly extrapolated to articular chondrocytes.

The primary purpose of a mouse model was to examine the effect of Epas1 deletion in the
development of osteoarthritis following meniscus destabilization in an effort to replicate human
knee injury. While the model successfully induced osteoarthritis and meniscus is destabilized via
the meniscotibial ligament, it does not replicate common human meniscus injury patterns. In the
mouse, the meniscus is hypermobile compared to the human meniscus and the joint biomechanics
are different. An additional limitation is that it is a rapidly progressive model of arthritis in mice,
which may not parallel human biology even though arthritis is accelerated after joint injury.
Despite these deficiencies, it does imply a role for the meniscus in the development of
osteoarthritis. The previous studies did not analyze HIF-1α in the context of HIF-2α protein localization in wild-types or Epas1 heterozygous knockouts. To better characterize potential alterations in the mouse joint, cartilage and meniscus should be stained for the presence of both HIF proteins. Staining for HIF-1α in addition to HIF-2α, would identify successful decrease in HIF-2α in Epas1 +/− mice and also better elucidate the role of HIF balancing in these tissues.

The regulation and influence of the HIF family genes is exceedingly complex. Multiple factors have been demonstrated to influence both their expression and activation, including catabolic stress, hypoxia and normoxia, and tissue types. The balance between HIF-1α and HIF-2α is much more likely to be responsible for effecting significant transcriptional changes, and this balance is controlled by a number of factors, including oxygen tension, tissue type, catabolic stress, and perhaps most importantly by species. Furthermore, the role of HIFs in the meniscus may be more related to phenotype differentiation, based on the number of chondrocytic targets it has than meniscal degeneration. Much of the evidence that examined HIF-1α and HIF-2α together, identified that the HIFs were responsible for both anabolic and anti-catabolic effects. Our results are more congruent with the theory that the two HIFs are dynamic and are not primarily responsible for osteoarthritic changes in the meniscus. Future emphasis should be placed on exploring the potential repair functions in HIF mediated anabolism and anti-catabolism in both meniscus and cartilage tissue. While the HIF family is likely involved in osteoarthritis development, HIF-1α and HIF-2α may be more involved in protective mechanisms in the joint following catabolic stress rather than destructive processes.

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from the Gift of Hope Organ and Tissue Donor Network.

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Society for Sports Medicine (Stone) and the clinician scientist grant from the Orthopaedic
Research and Education Foundation (Stone). Additional support was received from the
NIH/NIAMS K08AR059172 (Ferguson) and NIH/NIAMS R37 AR049003 (Loeser).

Competing interests:
The authors have no competing interests to report. Funding sources disclosed above.
Figure 1: HIF protein staining in normal and osteoarthritic meniscus. Light microscopy views of HIF-1α and HIF-2α stained sections of normal (NL) and osteoarthritic (OA) meniscus. Protein staining was developed with DAB and counterstained with eosin. The percentage of positively stained cells was not different: HIF-1α normal (55.3% ± 2.6%, n=2), osteoarthritic (75.3% ± 11.7%, n=5); HIF-2α: normal (75.4% ± 10.3%, n=2), osteoarthritic (83.9% ± 13.7%, n=5). Results reported ± standard deviation.
Figure 2: Relative expression of HIF genes in normal and osteoarthritic meniscus cells.

Samples are control groups used from meniscus cell stimulation experiments. (A) *HIF-1α* gene expression. (B) *Epas1* gene expression. Basal expression did not differ between normal and osteoarthritic cells at either time point. (Group: p=0.409; Time p=0.285). (C) Ratio of HIF gene expression in normal and osteoarthritic meniscus cell. No significant differences between groups or time point (group: p=0.669; time: p=0.331).
Figure 3: HIF expression in osteoarthritic meniscus cells following pro-inflammatory stimulation. Primary cell cultures were stimulated with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), or TGF-α (20 ng/ml) and cells were harvested 24 hours after stimulation (n≥4 unique donors). Differences between stimulation were not significant [HIF-1α (p=0.434); Epas1 (p=0.373)].
Figure 4: Effects of pro-inflammatory stimulation on HIF, MMP, and anabolic gene expression. Primary cell cultures were stimulated with IL-1β (10 ng/ml), IL-6 (10 ng/ml plus 25 ng/ml sIL6R), TGF-α (20 ng/ml) or fibronectin fragments (FnF, 1 μM) and cells were harvested 24 hours after stimulation (n≥4 unique donors). (A) HIF gene expression. Differences between stimulation were not significant (HIF-1α, p=0.434; Epas1, p=0.373) (B) MMP gene expression. MMP1: IL-1β, p=0.002; IL-6, p=0.005; FnF, p=0.002. MMP3: IL-1β, p=0.003; FnF, p=0.001. (C) Anabolic gene expression. Col2a1: TGF-α, p=0.010; Sox9: FnF, p=0.0002). Gene expression of catabolic and anabolic factors did not correlate with alterations in HIF gene expression. All real-time PCR data was normalized to internal control (unstimulated) for accurate full change comparisons. Error bars represent 95% confidence intervals (n≥4 unique donors).
Figure 5: Effects of hypoestoxide on HIF expression. (A) HIF expression in Normal and osteoarthritic meniscus primary cell cultures treated with the NF-κB inhibitor, hypoestoxide (HE, 25 μM) for six or 24 hours. HE significantly reduced HIF expression in osteoarthritic cells by 24 hours (p=0.023; n≥6 unique donors). (B) Effect of pro-inflammatory stimulation of normal meniscus cells in the presence of hypoestoxide (n=5 unique donors). Stimulation group effects were significant (p=0.036) and FnF increased HIF expression (p=0.009), but differences between HIF-1α and Epas1 were not significant (p=0.250). Error bars represent 95% confidence intervals. (FnF, fibronectin fragments)
Figure 6: Effects of adenoviral overexpression and knockdown on HIF-1α and HIF-2α proteins. (A) Immunoblot for HIF proteins following either null, HIF-1α, Epas1 (HIF-2α) adenovirus infection. The nuclear fractions contain the respective active transcription factor. LaminB was used as a loading control for nuclear (N) fractions. Lactate dehydrogenase (LDH) was the loading control for cytoplasmic (C) fractions.
Figure 7: Response of HIF family and target genes to HIF adenoviral overexpression or siRNA knockdown. (A) HIF family and developmental gene response to overexpression or knockdown. Adenoviral and siRNA treatment significantly altered the respective expression of
HIF-1α (p=0.009) and Epas1 (p<0.001). (B) Response of matrix metalloproteases. MMP expression is not significantly altered. (C) Response of collagen expression. Treatment group effects significantly affected Col1a1 expression (p= 0.014). Col1a1 was significantly reduced by Epas1 (p=0.044) but reduction by the HIF-1α virus was not significant (p=0.077). Treatment group effects were also significant in Col2a1 expression (p= 0.042), but Epas1 virus did not reach significance (p=0.075). Error bars represent 95% confidence intervals (n=4 unique donors).
Figure 8: Representative histology and analysis of following destabilized medial meniscus surgery in mice. (A) Medial compartment of wild type and Epas1+/- mice four weeks after DMM surgery. Orange arrows identify focal cell death with cartilage thinning. Black arrow demonstrates cartilage fissure with cell drop-out. (B) Articular cartilage structure (ACS) score and meniscus area from the medial compartment. No significant difference existed between groups.
Table 1: Correlation of HIF expression with selected catabolic and anabolic genes in normal meniscus following pro-inflammatory stimulation.

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<th>Col1a1</th>
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**. Correlation is significant at the 0.01 level (2-tailed).
Table 2: Correlation of selected genes following HIF-1α and Epas1 overexpression and knockdown in normal meniscus cells.

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<th>Mmp13</th>
<th>Col1a1</th>
<th>Col2a1</th>
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*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).
Supplementary Information

Figure S1: Genotyping of mice for Epas1 insufficiency. The wild-type (WT) allele appears at 222 base pairs and the mutant Epas1^{tm1Rus} allele appears at ~400 base pairs. The Epas1 heterozygote (Epas1^{+/--}) appears with both alleles.
Figure S2: Immunofluorescent staining of normal and osteoarthritic meniscus for HIF proteins. Fluorescent microscopy of HIF-1α and HIF-2α stained sections of normal (NL) and osteoarthritic (OA) meniscus. The nucleus was counterstained with DAPI.
Table S1: Grading system for cartilage and meniscus specimens.

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<th>Meniscus Adaptation</th>
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<td>Normal meniscus</td>
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<td>Near-normal with minor fibrillations</td>
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<td>Small, scattered cracks or fissures, some fibrillation</td>
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<td>Lesions &gt;50%</td>
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<td>Grade 4</td>
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Table S2: Articular cartilage and meniscus measurements in mice following DMM surgery.

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References

2. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) 2009; 2497-106.


4. Duval E, Leclercq S, Elissalde J-M, Demoor M, Galéra P, Boumédiene K. Hypoxia-inducible factor 1α inhibits the fibroblast-like markers type i and type iii collagen during hypoxia-induced chondrocyte redifferentiation: Hypoxia not only induces type ii collagen and aggrecan, but it also inhibits type i and type iii collagen in the hypoxia-inducible factor 1α–dependent redifferentiation of chondrocytes. Arthritis & Rheumatism 2009; 60(10): 3038-3048.


Discussion and Conclusions: The Meniscus in Osteoarthritis

Austin V. Stone, MD
**Discussion and Conclusions**

The clinical importance of the meniscus in osteoarthritis pathogenesis is well documented and the burden of meniscus injuries and osteoarthritis development will continue to increase\(^1\)-\(^{21}\). The options for treating meniscus injury as orthopaedic surgeons are limited because of limited healing in the avascular zones and limited understanding of meniscus pathobiology\(^9\)-\(^{11}\), \(^{19}\), \(^{22}\), \(^{23}\). Meniscus pathology in osteoarthritis is largely attributed to mechanically mediated loss of structural integrity\(^1\), \(^6\), \(^{16}\), \(^{24}-^{26}\). These biomechanical stress factors may lead to “osteoarthritis in the meniscus” which is proposed to be responsible for meniscus MRI changes observed during the early osteoarthritis development\(^7\). Our data supports and expands upon recent evidence that the meniscus may have a more biologically active role in the complicated whole-joint pathology of osteoarthritis\(^22\), \(^{27}-^{31}\).

We investigated meniscus involvement in the natural history of osteoarthritis in vervet monkeys. Vervet knees were demonstrated to have osteoarthritic changes that were similar to humans and consistent with established reports of spontaneous and surgically induced osteoarthritis in non-human primates\(^32\)-\(^{36}\). A pilot project at our institution identified osteoarthritic changes in the vervet shoulder including glenoid retroversion and joint space narrowing\(^36\). Our cross-sectional analysis suggested that pathologic degradation of the meniscus corresponded to more severe bony changes and cartilage degradation. Meniscus degenerative changes were previously correlated with osteoarthritic changes in cartilage and articular cartilage loss\(^9\), \(^{37}\), \(^{38}\). In a small human magnetic resonance imaging (MRI) study, meniscus deterioration accompanied or preceded severe articular cartilage loss\(^37\). A larger MRI study identified an increasing risk of cartilage loss with increasing number of meniscus abnormalities\(^38\). We identified similar correlations between meniscus and cartilage morphologic scores and found that higher degeneration scores were associated with a greater production of matrix metalloproteases (MMPs) and cytokines.
Increased production of MMPs, cytokines and chemokines are believed to be responsible for propagating the reactive responses and degradation in joint tissues that ultimately lead to osteoarthritis. In our study, aged and degraded vervet menisci produced both increased MMPs and cytokines compared to younger vervet menisci. Human meniscus cell cultures responded to pro-inflammatory stimulation with increases in catabolic activity similar to those observed in osteoarthritic vervet menisci and complementary to MMP patterns observed in human osteoarthritic cartilage. Both human meniscus cells and degenerative vervet menisci demonstrated increased production of MMP1, MMP3 and MMP8 in response to pro-inflammatory stimuli. These alterations in MMP expression are important in osteoarthritis development and progression. MMP1 degrades collagen type I which is the primary constituent of meniscal extracellular matrix. Increased MMP1 activity likely damages the structural integrity of the meniscus, which may in part explain the gross fibrillation and fissuring seen at the time of osteoarthritis meniscus specimen acquisition. Pig meniscus explants demonstrated increased MMP1 activity, proteoglycan release and nitric oxide release in response with IL-1 stimulation. MMP3 cleaves multiple matrix proteins and activates other MMPs, including MMP1. MMP3 (stromelysin-1) production is similarly important because it is upregulated in articular cartilage in early osteoarthritis, and MMP3 was increased with aggrecanases in partial meniscectomy specimens. MMP8 expression was increased in stimulated normal human meniscus cells and MMP8 production was elevated in aged and degenerative vervet meniscus. MMP8 activity in osteoarthritis pathogenesis is less well studied, but it is capable of degrading collagen, is associated with neutrophil infiltration, and co-localizes with IL-1β and type II collagen cleavage in osteoarthritic cartilage. MMP10 expression was increased with pro-inflammatory stimulation in normal meniscus cells. MMP10 was reported in the fibrocartilaginous nucleus pulposus and was associated with increased gross and histological degeneration, pain, and increased IL-1 and substance P.
Pro-inflammatory stimulation also increased MMP-13 gene expression and production in normal meniscus cells. Our findings are consistent with increased MMP-13 following IL-1α treatment in normal inner meniscus and articular chondrocytes. Increased MMP-13 gene expression in stimulated normal meniscus cells is also congruent with reported MMP-13 expression in partial meniscectomy specimens. The inner region of the meniscus would be expected to constitute the majority of cells removed in a partial meniscectomy. The meniscus cell phenotype is reported to become increasingly chondrocytic in the inner zones of the meniscus. The inner section is likely the first section to deteriorate in osteoarthritis progression and may explain in part why we did not see significant increases in MMP-13 production in our osteoarthritic meniscus cells.

Meniscus cells can be stimulated to produce matrix degrading enzymes which may impact the nearby cartilage, but the tissue interaction is likely part of a more dynamic signaling network. Osteoarthritic vervet meniscus and stimulated human meniscus cultures displayed increased cytokine and chemokine expression and production in a manner similar to chondrocytes. Multiple interleukins were increased in both gene expression and protein production in stimulated normal human cells, including IL-1β and IL-6 that were used as pro-inflammatory stimuli. IL-6 secretion appeared to be elevated in older and higher grade vervet menisci but was also present in younger vervet menisci. Increased IL-6 production is implicated in osteoarthritis pathogenesis. The reason for elevated vervet IL-6 secretion is unclear, but IL-6 production in younger vervet menisci could be evidence of early pathology.

IL-7 was also shown to be produced by aged and degenerative vervet menisci and increased by IL-6 in human cell culture. In chondrocytes, treatment with fibronectin fragments or IL-1β and IL-6 increased IL-7 release. Chondrocyte stimulation by IL-7 was previously demonstrated to increase MMP-13 production, extracellular matrix degradation, proteoglycan release. The
Catabolic effects of IL-7 were demonstrated to occur through the inflammatory JAK3/STAT pathway that upregulated S1004A and MMP13 gene expression. Human synovial levels of IL-7 are increased in earlier stages of osteoarthritis, greater in older patients with osteoarthritis, and increased in patients with synovitis. Our findings are consistent with age and disease dependent increases in IL-7, which may also have a role in meniscus osteoarthritis.

The recognition of chemokines in osteoarthritis pathogenesis is growing. We identified increased IL-8 production in stimulated human menisci and small increases in older explants. While the role of IL-8 in osteoarthritis is not fully elucidated, IL-8 is thought to contribute to chondrocyte hypertrophy, calcification and crystals in the joint, and the development of subchondral bone sclerosis. Increased IL-8 expression was also identified in the synovium and canine cranial cruciate ligament [(analogous to the human anterior cruciate ligament (ACL)] in osteoarthritis development following ligament rupture. Chemokines CXCL1, CXCL2, CXCL3 (identified by the GRO antibody) were expressed in both young and old menisci, with a slight qualitative increase in older monkeys; however, in our human meniscus study, we were able to identify increased CXCL1, CXCL2, CXCL3, CCL8 (MCP-2), CCL7 (MCP-3) CXCL6 (GCP-2) with pro-inflammatory treatment. These chemokines may contribute to the development of inappropriate inflammatory cycles after injury, and interestingly, the GROα chemokines increase hypertrophic markers in chondrocytes. Our results support recently reported findings in an analysis of gene expression in meniscus tears, which found increased expression of IL-1β, ADAMTS-5, MMP-1, MMP-9, MMP-13, and NFκB2 in patients with meniscus tears younger than forty. Cytokine and chemokine expression (including IL-1β, TNF-α, MMP-13, CCL3, and CCL3L1) were greater in patients with a meniscus tear and concomitant ACL tear than those with only a meniscus tear, which suggests that a larger increase in catabolic activity may occur with a more severe injury. The catabolic activity identified in meniscectomy specimens supports our cell culture and vervet models.
We identified a potential role for additional chemokines and cell adhesion markers in the meniscus. GM-CSF was increased in both degenerative vervet menisci and stimulated human cells accompanied by an increase in Lymphotoxin β expression in human cells. Although both chemokines are primarily linked to rheumatoid arthritis, they are also reported in the osteoarthritic synovium. These chemokines may be linked to the more fibroblastic cell phenotype of the meniscus or to inflammatory cell recruitment. Catabolic expression was accompanied by a notable decrease in expression of the anabolic factor CTGF. CTGF was recently identified in a rabbit model for promoting collagen production and healing of meniscus defects. The combination of abnormal inflammatory cell recruitment and decreased anabolic factors could easily compromise wound healing.

Human meniscus cells demonstrated altered expression of cell adhesion proteins in response to pro-inflammatory stimulation. Alterations in the meniscus integrin receptor expression were previously identified in osteoarthritis meniscus cells and would be expected to alter cell-matrix interactions in a manner similar to chondrocytes. Cell adhesion markers VCAM-1, ICAM-1 and E-selectin were also increased and were previously demonstrated to be present in hypertrophic and early osteoarthritic synovium and are involved in inflammatory cell recruitment to the synovium. ICAM-1 was specifically identified as increased in early osteoarthritis and ICAM-1 expression was increased in the development of osteoarthritis following canine cranial cruciate ligament rupture. VCAM-1 was shown to be predictive of joint replacement for severe arthritis. Pharmacologic reductions ICAM-1 and VCAM-1 in early to mid-stage knee osteoarthritis were associated with pain and functional improvements.

We observed age related increases in inflammatory cytokines and MMP production in the natural history of osteoarthritis in vervets and expanded that analysis in human normal and osteoarthritic meniscus cells. Cytokine and matrix degrading enzyme production can be assumed
to act on local tissues. In the case of the meniscus, the secretion of enzymes and cytokines are likely acting on both cartilage and the synovium to stimulate catabolic processes. This mechanism for joint destruction is supported by a number of studies identifying these factors as increased in the disease state and detailing their deleterious effects on cartilage, bone and the synovium \cite{28,39,43}, which would likely suppress reparative cell functions and propagate a loss of matrix integrity. This concept may be one way the meniscus contributes to osteoarthritis development, especially after meniscal injury, and these findings may better explain the higher failures in meniscus repair in older patients\cite{3,21,22}. The production of pro-inflammatory factors and matrix-degrading enzymes may support a role for biologic activity of the meniscus in the development of osteoarthritis in regions of the joint that do not contact the meniscus. If an insult to the cartilage of another region of the joint, such as the patellofemoral joint, released pro-inflammatory factors into the synovial fluid, then these factors may act on the meniscus cells to increase the secretion of cytokines, chemokines and matrix-degrading enzymes. The meniscus may be a biologic contributor to the whole joint pathology of osteoarthritis.

The MMP, cytokine, and chemokine genes first evaluated in vervet explants and then in human cell culture are targets of the nuclear factor-κB (NF-κB) pathway in chondrocytes\cite{52}. We found IL-1β with IL-6, and FnF stimulation to increase expression and production of NF-κB and its targets and we demonstrated that pro-inflammatory stimulation increased phosphorylation of the NF-κB subunit p65. Inhibition of the NF-κB pathway successfully decreased NF-κB family gene expression and reduced production of MMP1 and MMP3 in human meniscus cells. The effects of inhibiting IL-1β and IL-6 in our studies were less profound since IL-6 is known to act through pathways other than NF-κB\cite{61}. FnF had the most profound effects on cytokine and chemokine production, which is not surprising given its strong induction of the NF-κB pathway in chondrocytes\cite{28,39,48,62}.
The NF-κB pathway has been extensively studied in chondrocytes; however, the data is limited for meniscus cells. A study of partial meniscectomy specimens identified increased phosphorylated-p38 (MAP kinase activity) and phosphorylated-p65 (NF-κB pathway activity) in more degenerated and osteoarthritic meniscus specimens\textsuperscript{18}. The increased phosphorylated-p38 and –p65 staining was associated with an increased in pro-inflammatory cyclooxygenase(Cox)-2 staining\textsuperscript{18}. The upregulation of NF-κB chemokine target expression in patients with meniscus tears discussed earlier, was accompanied by an upregulation of NF-κB\textsuperscript{22}. This finding suggests that activation of the NF-κB pathway occurs in meniscus injury. The catabolic effects in our studies appear to be mediated, at least in part, by activation of the NF-κB pathway.

Investigators only recently began to consider joint as an organ with complex interactions that are much greater than the sum of its parts. The biology of the injured meniscus and the subsequent catabolic and repair responses provides many exciting opportunities for therapeutic intervention; however, the basic biology of the meniscus and its role in osteoarthritis remains in early stages of exploration. The responses of the meniscus and its similarities to cartilage catabolism through NF-κB activation suggested that similar mechanisms may be active in the meniscus. The hypoxia-inducible factor (HIF) pathway is linked to the NF-κB pathway and may offer insight into joint tissue catabolic responses.

**The role of the HIF pathway**

Hypoxia-inducible factors are widely studied in an array of cell types\textsuperscript{63-69}, but only recently became a subject of exploration in joint tissues\textsuperscript{70-81}. Two studies implicating HIF-2α in osteoarthritis pathogenesis in articular cartilage provided the foundation for our HIF studies in the meniscus\textsuperscript{78, 81}. Since the meniscus is involved in osteoarthritic development and meniscus cells share phenotypic similarities with articular chondrocytes, we hypothesized that upregulation of HIF-2α would be responsible for catabolic increases in osteoarthritic alterations in the meniscus.
Differences in HIF responses

The upregulation of HIF-2α and its gene Epas1 was reported to be an important novel pathway in osteoarthritis pathogenesis. A series of experiments in mice and human cell cultures linked its upregulation to increased catabolism in articular cartilage, however, the dynamics of HIF regulation and response are complex (Table 1). HIF-1α is conserved from C. elegans to humans; HIF-2α is conserved only in avians and mammals, which suggests that its role is not merely duplicative of HIF-1α, but that it is a necessary adaptation to the more complex structure of a larger biologic system. The balance between HIF-1α and HIF-2α is much more likely to be responsible for effecting significant transcriptional changes, and this balance is controlled by a number of factors, including oxygen tension, tissue type, catabolic stress, and by species.

Epas1 knockdown in our mouse destabilized medial meniscus (DMM) model did not prevent osteoarthritic changes, which were evident within four weeks of DMM surgery. Our results are in contrast to the chondro-protection reported by Yang et al., although they reported an eight week time point. Mice in our study had similar osteoarthritic changes and similar meniscus histologic assessment at both the two and four week time points. Our study included a larger number of mice at each time point (Stone et al n≥13 versus Yang et al. n=981) and a more rigorous histologic score with tightly grouped data. The studies of HIF-2α in osteoarthritis development did not examine HIF-2α in the context of HIF-1α; they explored only HIF-2α translation and localization in Epas1+/− DMM mice and mice overexpressing Epas1. As Murphy suggested, the lack of HIF-1α data, the excessive synovitis noted with HIF-2α adenovirus intra-articular injection, and the lack of demonstrable cartilage-specific Epas1 recombinant expression may lead to an unreliable model and skewed results. Furthermore, the HIF-2α target genes were primarily defined in mouse articular chondrocytes and not in primary...
healthy human chondrocytes. These experiments were all completed without monitoring resultant HIF-1α changes.

In human meniscus cell culture, we opted to evaluate both HIFs since previous literature most frequently evaluated either HIF-1α or Epas1 and since their effects are likely exerted in concert. We did not observe differential production of HIF-1α and HIF-2α in normal and osteoarthritic meniscus tissue, which was different than reported in osteoarthritic human and mouse cartilage, but congruent with other reports of HIF expression in human normal and osteoarthritic chondrocytes. Our findings may be related to cell culture conditions where cells are removed from their native environment. To better evaluate meniscus HIF responses, cell culture were treated with pro-inflammatory stimulation previously demonstrated to affect HIF-1α and HIF-2α.

Pro-inflammatory stimulation did not dynamically alter HIF expression. Treatment of meniscus cells with the cytokines IL-1β and IL-6 did not significantly increase HIF-1α or Epas1 expression, which was unexpected since cytokines were previously reported to increase Epas1 expression in mouse and rabbit chondrocytes and human cell lines. Interestingly, FnF increased relative Epas1 and HIF-1α expression. Yang et al. stimulated cultured mouse and rabbit articular chondrocytes with IL-1β (5ng/ml) and Epas1 expression was increased by 6 hours through 36 hours after stimulation with an increase in HIF-2α protein by 6 to 12 hours. Saito et al. demonstrated increased Epas1 expression two days after IL-1β (1ng/ml) stimulation in chondrogenic cell lines. Increased expression with increased protein levels would likely lead to increased HIF-2α activity. Since our IL-1β concentration (10ng/ml) was greater than both reports, we would expect to see an increase in Epas1 expression in meniscus cells. Yang et al. described Epas1 induction by multiple pro-inflammatory cytokines, including TNF-α, IL-1β, IL-17 and IL-21, but did not place these results in the context of HIF-1α which is also known to be increased.
by pro-inflammatory stimulation. Catabolic stress, including IL-1β, was responsible for inducing HIF-1α expression in osteoarthritic chondrocytes and was upregulated in more damaged regions of cartilage.

Downstream transcription target genes of the HIF pathway were incongruent with a primarily catabolic effect of Epas1. HIF-1α expression positively correlated with Epas1 in stimulated cells, which may suggest a common pathway of induction or synergistic effects. Following pro-inflammatory stimulation, both HIF-1α and Epas1 positively and significantly correlated with MMP1 and MMP3 expression. Reported targets of Epas1 in osteoarthritic chondrocytes include MMP1 and MMP3, in addition to MMP9, MMP12 and MMP13; however, the correlation observed in our meniscus cells may be attributable to a HIF-independent pathway. Neither MMP1 nor MMP3 correlated with Epas1 overexpression, while HIF-1α overexpression exhibited a significant negative correlation with MMP3 expression. Negative correlation of HIF-1α overexpression with MMP3 parallels the reported down regulation of MMP13 by HIF-1α. Furthermore, we observed significant increases in MMP expression in meniscus cells by six and twenty-four hours, which overlapped the maximal levels of Epas1 expression and HIF-2α production in rabbit and mouse chondrocytes of Yang et al.; consequently, the effects of downstream targets of Epas1 would be expected to be apparent by this point. In chondrocytes and meniscus cells, MMP increases are linked to multiple inflammatory pathways, including NF-κB. MMP1 and MMP3 are likely not downstream targets of the HIF pathway in human meniscus cells.

We did observe a decrease in both HIF-1α and Epas1 expression following NF-κB inhibition in osteoarthritic meniscus. The decrease is consistent with previous descriptions of NF-κB regulation, since both genes have been shown to have stabilized or upregulated at least in part through the NF-κB pathway. While HIF-1α and Epas1 do have interactions with the
NF-κB pathway, the decrease in HIF expression following NF-κB inhibition may be the result of inhibiting a larger cellular repair response in diseased or stressed tissue and less of a sole consequence of NF-κB activation. Saito et al examined a number of transcription factors known to regulate chondrocyte differentiation and found that the NF-kB p65 (v-rel reticulendotheliosis viral oncogene homolog A (RELA)) to be the strongest activator of the Epas1 promotor fragment in human chondrogenic cell lines and found that HIF-2α co-localized with RELA in mice. They did not perform experiments where Epas1 (and HIF-2α) were examined in the context of inflammatory pathway inhibition. Yang et al. did perform some pathway inhibition experiments in mice but these were limited in their scope. Yang et al. used Bay 11-7085, which is known to have p38 effects and significant cytotoxicity at concentrations as low as 5-10 μM. Bay 11-7085 may have affected gene expression through cytotoxicity, and thus the results of Yang et al may be compromised. Hypoestoxide was used as an NF-κB inhibitor since it does not have cytotoxic effects and inhibits NF-κB action in chondrocytes. The link between HIF-2α and NF-κB remains unclear. It is possible that HIF-2α is associated with the NF-κB pathway as a repair response. Although NF-κB pathways have a definitive role in inflammation, NF-κB has also been identified in some tissues as aiding in inflammatory resolution and tissue repair. The HIF pathway may constitute a protective pathway under catabolic stress.

A protective response to catabolic stress?

While alterations in the HIF pathway did not have a clear relationship to destructive osteoarthritic changes in the meniscus, the HIF pathway may be important in initiating protective pathways following catabolic stress and directing cell phenotype. In previous studies, HIF-1α and HIF-2α were demonstrated to control energy production and share many of the same targets which was theorized to improve tissue repair. We found that HIF-1α and Epas1 expression was positively correlated with Sox9 and collagen type II following
pro-inflammatory stimulation in normal meniscus cells. In our studies, Sox9 significantly and positively correlated with collagen type II and aggrecan. Epas1 overexpression also increased collagen type II expression. Sox9 is an important developmental gene found to be responsible for the phenotypic development of both meniscus and cartilage cells and upregulates chondrocytic matrix genes, including Col2a1, Col9a1, Col11a2 and aggrecan\textsuperscript{72, 90}. Collagen type II is also present in the chondrocytic inner zones of the meniscus\textsuperscript{44, 100, 101}.

The regulation of Sox9 in both meniscus cells and articular chondrocytes is unclear. HIF-1\textalpha{} has been demonstrated to have a significant role in Sox9 regulation but much of this work was either completed in osteoarthritic cartilage and meniscus or in the mouse\textsuperscript{76, 86, 99}. Additional studies identified that human articular cartilage responds differently from that of mice\textsuperscript{84, 89, 90}. Sox9 induction was dependent on HIF-2\textalpha{} and not on HIF-1\textalpha{} in human articular chondrocytes\textsuperscript{72}. Increased HIF-2\textalpha{} in human chondrocytes is linked to increased Sox9 expression and increased collagen type II and aggrecan\textsuperscript{72, 90}, which is consistent with our findings in the meniscus. In osteoarthritic meniscus cultures where inner and outer meniscus cells were separated, HIF-1\textalpha{} was associated with increased Sox9 and collagen type II in outer meniscus cells but had no relationship to Sox9 in inner (more chondrocytic) meniscus cells\textsuperscript{86}. HIF-2\textalpha{} is also linked to the control of additional anti-catabolic responses in healthy human articular chondrocytes through upregulation of Sox9 independent genes, chondrocyte regulators Mig6 and inhibin A (InhbA), cartilage matrix protein chondromodulin-1 (Chm-1), and cartilage growth factor Gdf10\textsuperscript{71}. Mig6 is linked to tissue maintenance in chondrocytes. Both HIF-1\textalpha{} and HIF-2\textalpha{} upregulate inhibin A which prevents MMP activation by increasing tissue inhibitor of matrix metalloproteinases (TIMP-1)\textsuperscript{71}. These findings increasingly support the notion that the HIF activity may be induced by catabolic stress, but respond with an anabolic and anti-catabolic response.
Another potential anabolic role for HIF-1α in the meniscus may be for regulation of vascular in-growth. VEGF is a known target of both HIF-1α and HIF-2α, but HIF-1α may be a more potent regulator of VEGF expression\textsuperscript{64, 77}. We observed increases in VEGF with HIF-1α and Epas1 overexpression. Fibroblastic cells of the outer meniscus may express HIF-1α, which is known to produce an anabolic response through metabolic upregulation and to generate both collagen and vascular in-growth\textsuperscript{73}. Chondromodulin-1, a target of HIF-2α in chondrocytes\textsuperscript{71}, was recently identified in the inner zones of the meniscus\textsuperscript{102}. Chondromodulin-1 is thought to counteract the angiogenic functions of VEGF to maintain avascular tissues\textsuperscript{73, 102}. This balance may benefit maintenance of the meniscus cell phenotype; however, our ability to resolve HIF-1α and Epas1 relationships to VEGF was limited by the heterogenous phenotype of our cell cultures.

The role of the HIF pathway in the development and maintenance of human joint tissues is extraordinarily complex and appears to be influenced by cell type and species. Evidence to date suggests a great deal of transcription target overlap with a delicate balance for activity and conflicting evidence on regulatory pathways. Recurrent themes of the HIF pathway include its potential involvement in cell protection and tissue repair in addition to contributing to cartilaginous cell phenotype. These findings make the HIF pathway a prime target for future research. A detailed analysis of HIF activity may be warranted to delineate transcription target genes, tissue-specific variances, and hypoxic mechanisms in joint tissue development.

Manipulation of the HIF pathway holds promise for meniscus cell differentiation and meniscus repair.

**Limitations**

Our investigation carries many common limitations of laboratory and animal models. Vervet monkeys underwent CT scanning to optimize whole body visualization, and consequently, variations in extremity positioning allowed for only qualitative assessments of bony disease.
Although the osteoarthritic changes we observed are consistent with human disease, vervets are quadripedal and experience different forces across the joint which may predispose them to different injury or disease patterns than humans. The cost and complexity of maintaining vervets allowed us to examine only two age cohorts of elderly and middle aged vervets, which did not document the full progression of meniscus disease in naturally occurring osteoarthritis. Finally, we examined explant secretion but did not perform protein, RNA or immunohistochemical analysis on the cultured explants. Future studies may examine the tissue expression patterns in the vervet meniscus.

Our human studies utilized cell culture models to study the osteoarthritic meniscus and normal meniscus. Cell culture responses may not directly mimic those of the intact meniscus in its native environment, but the use of primary cell culture provided an effective modality for precise control of stimuli and measurement of gene expression and protein production. Secreted products in the media were assumed to be available to act on local tissues and to be present in the synovial fluid. We did not examine which cells were producing these factors in the intact meniscus; however, the vervet explant studies did demonstrate that the intact meniscus explant released proteins into the media. We operated under the assumption that the MMPs would be active in the extracellular space but did not assess MMP activity. Another limitation of our cell culture studies is that we examined the meniscus cell population as a whole. The meniscus is comprised of both chondrocytic and fibroblastic cell phenotypes, and our experiments did not differentiate between inner versus outer meniscus cells. It is possible that a dominant cell response may mask that of the other cell types. The mixed cell population may confound the HIF responses, especially if a primary role of the HIF family is to promote cell anabolism and chondrocyte differentiation. Conflicting results for HIF behavior of inner versus outer meniscus cells and fibroblasts further complicates our interpretations. Results in the meniscus cannot be directly extrapolated to the behavior of articular chondrocytes.
Cell culture studies also carry the limitation that the cells are not in their native environment. In our monolayer culture, cells did not experience the compressive strains present in the native meniscus that may either aid in repair or be detrimental to meniscus biology. Static biomechanical loading is well established to be detrimental to chondrocyte biology, but cyclic loading can increase chondrocyte anabolic gene and protein expression. The meniscus response to biomechanical loading is less well defined. Dynamic loading of meniscus explants is reported to increase proteoglycan synthesis and protein synthesis, while static loading or overloading can result in increased MMP1 levels and reduced type I collagen expression. Increased loading can also lead to increased effects of IL-1 and MMP production.

Biomechanical loading may in turn be detrimental through the combined cell response to the catabolic effects of cytokines in the meniscus, which can inhibit or weaken the biomechanical strength of potential repairs. Catabolic activity, including MMP1, MMP3, MMP9, and MMP13, was also previously reported to be increased with compressive strain in explants. Inhibition of pro-inflammatory signaling in meniscus explants subject to compressive strain reduced catabolic expression, including multiple MMPs. By eliminating the biomechanical component of meniscus injury and repair, we were successful in evaluating some pro-inflammatory cell responses, but may be limited in our translation of the biochemical cell responses in the native meniscus.

In addition to cells cultured in monolayer, our cell culture studies were performed in normoxia, which would impact oxygen sensitive gene expression. Previous reports suggested that HIF proteins are stabilized by pro-inflammatory stimulation and that HIF expression was still present and active under normoxic conditions. While HIF expression is reported to be influenced by cytokines, much of the regulation may occur at the protein level and not the expression level. Proteosome degradation of the HIFs is well described, but its methods for activation and not just degradation appear complex. Additionally, HIF levels can be heavily
influenced by prolyl hydroxylation as demonstrated in some studies\textsuperscript{84, 89, 90}, and may not be stabilized by inflammatory stimulation to the degree originally proposed in recent studies\textsuperscript{78, 81}. Specific sequences in the HIF genes also play a role in protein binding and activation, so the sensitive balance between the two actually supports a delicate quantitative regulation more so than a simple qualitative relationship\textsuperscript{85}. The sensitivity in HIF regulation likely accounts for the reciprocal changes in HIF-1α and Epas1 expression following viral infection.

We did not explore the effects of hypoxia on HIF activity, since HIFs were reported to be present and active in normoxia\textsuperscript{81-83}, but hypoxia can increase expression of multiple genes associated with metabolic adaptation to low oxygen in many cell types\textsuperscript{68, 69}. Hypoxia enhances HIF-1α activity in mouse and bovine chondrocytes to maintain metabolic homeostasis and increase extracellular matrix synthesis\textsuperscript{70, 75}, and in human chondrocytes, hypoxia increases HIF-2α activity to maintain the chondrocyte extracellular matrix\textsuperscript{71, 72, 74, 90}. HIF-1α in meniscus cells was also increased in hypoxia, but the target gene relationship appeared to vary by meniscus region\textsuperscript{86}. In fibroblasts and osteoarthritic fibrochondrocytes, hypoxia increased cell motility and influenced collagen expression when exposed to different cell culture matrices\textsuperscript{103, 104}. Hypoxia and media composition also directed chondrocytic phenotypes in human bone derived and adipose derived mesenchymal stem cells\textsuperscript{114, 115}. Mesenchymal stem cell proliferation was actually enhanced in hypoxia with reduced oxygen damage\textsuperscript{115, 116}. Both an increase in collagen deposition and the protection from hypoxic conditions was attributed to HIF-2α (the authors did not examine HIF-1α which was likely also upregulated)\textsuperscript{115}. Increases in collagen type II expression were observed and positively correlated with Epas1 in our experiments, although we did not observe the same trend with collagen type I.

Epas1 insufficiency in our mouse model was not consistent with the chondroprotective effects reported by Yang et al., Siato et al, and Ryu et al.\textsuperscript{78, 81, 83}. We utilized wild-type
littermates to compare to Epas1\(^{+/+}\) mice, as did Siato et al\(^{78}\), but it is unclear if Yang et al. used another C57B6 mouse strain for the control. Strain differences result in different propensities for developing osteoarthritis\(^{117}\). Technical adequacy of the DMM surgery were confirmed by osteoarthritic changes by four weeks; early osteoarthritic changes were both temporally and histologically congruent with previous reports\(^{118,119}\). Our histologic grading system was more rigorous than that used for prior Epas1\(^{+/+}\) mouse studies and consequently identifies tissue specific and more subtle early disease alterations that may not be evident with the Mankin scale\(^{118,119}\). It is possible that the chondroprotective effects of Epas1 insufficiency may become more evident after four weeks, but we do not have a seven week time point to compare to Yang et al.\(^{81}\). An additional limitation in our model is that we did not examine protein production or localization of either HIF-1\(\alpha\) or HIF-2\(\alpha\). Immunohistochemical analysis could identify a successful decrease in HIF-2\(\alpha\) in Epas1\(^{+/+}\) mice and also better elucidate the role of HIF balancing in these tissues. The balance in HIF activity may include a delayed healing response with increases in HIF-1\(\alpha\) activity or potentially a halt in disease progression in the Epas1\(^{+/+}\) mice. While the DMM model successfully induced osteoarthritis, it does not replicate common human meniscus injury patterns. Mouse knee biomechanics are different than those of humans and mouse menisci are hypermobile compared to human menisci. An additional limitation is that it is a rapidly progressive model of arthritis in mice, which may not parallel human biology despite accelerated osteoarthritis after joint injury.

**Future Studies**

The primary purpose of the vervet meniscus explant and the human cell culture studies were to explore the pathobiology of the meniscus in response to both pro-inflammatory stimulation associated with injury and the development of osteoarthritis. As aforementioned, we did not look at the inner and outer zones of the meniscus separately. The role of HIF proteins in the response to injury may be related to cell phenotype and may direct both the inflammatory and healing
processes. Fuller et al. demonstrated different responses of inner versus outer meniscus to cytokine stimulation\textsuperscript{29}. A more comprehensive characterization of the inner and outer meniscus cell response to pro-inflammatory stimulation may better clarify MMP expression patterns and provide information that may be used to better target therapies for meniscus tears or degenerative meniscus. Inner and outer zones of osteoarthritic meniscus demonstrated differential HIF-1\(\alpha\) and prolyl hydroxylase expression\textsuperscript{86}. The limited number of samples in the osteoarthritic tissue precludes conclusions about the role of meniscal HIFs\textsuperscript{86}, but HIF responses to pro-inflammatory stimulation should be characterized in inner versus outer meniscus to clarify the HIF role in the meniscus metabolism. Additionally, the role of HIF-3\(\alpha\) was also recently implicated in osteoarthritis and may additionally regulate HIF-1\(\alpha\) and HIF-2\(\alpha\)\textsuperscript{120}. The authors identified that proteoglycan 4 (Prg4) upregulated HIF-3\(\alpha\), which in turn, down-regulated HIF-1\(\alpha\) and HIF-2\(\alpha\) in mice. Suppression of chondrocyte hypertrophy and catabolic activity was suggested by the findings that overexpression of Prg4 resulted in increased HIF-3\(\alpha\) expression with decreased VEGF, collagen type X, and MMP13 expression\textsuperscript{120}. The potential role of HIF-3\(\alpha\) in protecting against catabolic responses is intriguing, but additional exploration is warranted since HIF-3\(\alpha\) was reported as minimally expressed in chondrogenic differentiation of mouse ATDC5 cells\textsuperscript{78}.

Another strategy for examining the role of the HIF pathway in murine joints is through tissue specific overexpression or knockdown of the HIF proteins. Yang et al used the collagen type II driven expression of Epas1, but the degree of overexpression was questionable secondary to unreliable collagen type II expression at that age\textsuperscript{81, 84}. The use of an aggrecan promoter may be more efficacious in driving overexpression or knockdown of HIF-1\(\alpha\) and Epas1, since aggrecan was identified as consistently expressed in both the meniscus and cartilage in young and old mice\textsuperscript{121}. Collagen type X was also identified in both young and old mice, but is a marker of human hypertrophic chondrocytes and usually not in healthy adult articular cartilage or widely expressed in the meniscus\textsuperscript{52, 121}. The promoter may also be engineered to overexpress siRNA
specific for one or both HIF genes. Furthermore, conditional knockouts could be generated to selectively eliminate one or both HIF genes that could be accomplished in a tissue- and time-specific fashion\textsuperscript{122, 123}. This strategy would allow the potential role of HIFs in tissue repair to be explored. Future experiments should also analyze HIF-1α in the context of HIF-2α production and localization in wild-types and transgenics to evaluate for compensatory changes in HIF expression or activity.

In addition to examining both HIF-1α and HIF-2α expression and activity in the murine joints, an eight week time point may also be added to the DMM experiments to provide a direct comparison to the previous HIF studies\textsuperscript{78, 81}. By eight weeks, it is possible that the HIF response may be involved in healing responses or further cartilage degradation. The effects of the HIF pathway may be to prevent further degradation or potential reversal of the degradation. Protective responses of HIF-1α may be evident over time secondary to accumulation of the protein with the reduction of HIF-2α activity. To examine these possibilities, in addition to HIF protein staining, one could stain for HIF targets involved in degradation (such as MMP13 and MMP9) in addition to anabolic staining such as collagen type I and II, VEGF and aggrecan. If HIF-2α targets were decreased in the Epas1\textsuperscript{+/−} mice, the anabolic effects of HIF-1α may become more pronounced and contribute to the repair of the tissue that had its initially destructive HIF-2α halted. An increase in these anabolic targets and mid and late time points in the DMM Epas1\textsuperscript{+/−} mice may support a delayed healing response that would be evident at eight weeks.

Our meniscus cell data is not congruent with the responses reported by Yang et al.\textsuperscript{81} and Siato et al.\textsuperscript{78}, but that may be a function of differences between meniscus and cartilage. To confirm that the role of HIF-2α is not primarily catabolic in human joint tissue and that our results are not solely a function of meniscus cell phenotype, the meniscus experiments should be duplicated in primary chondrocytes. The target genes in the stimulation analyses should include MMP13, HIF
targets VEGF and aggrecan, and potentially HIF-linked chondrocytic genes, such as Mig6, Chm-1, and inhibin A (InhbA) to confirm the previously reported HIF targets\textsuperscript{71}. To better define potential target genes of the HIFs, pro-inflammatory stimulation experiments may also be strengthened by stimulating cells in the presence of siRNA specific to HIF-1α, Epas1 or both. A second factor to consider is that our experiments were designed under the premise that catabolic stress through pro-inflammatory stimulation would stabilize HIF-2α and lead to its catabolic effects\textsuperscript{78, 81, 83}. HIFs were previously reported to be active in normoxia\textsuperscript{75, 77, 90}, however, cell responses were more dynamic in reduced oxygen tension and the initial results of the meniscus pro-inflammatory stimulation experiments may be skewed by normoxic conditions\textsuperscript{71, 72, 89, 90}. Viral experiments may still be acceptable to be duplicated in normoxia since the HIF pathway will be saturated and HIF proteins were identified in the nucleus where target genes are activated\textsuperscript{124}. In light of our studies and the literature published since commencing our studies\textsuperscript{89, 90, 103, 125, 126}, hypoxic cultures may better replicate the native joint environment and may help elucidate the mechanism for HIF anabolic and anti-catabolic responses in addition to its effects on cell differentiation.

While mouse models offer a great degree of flexibility for genetic studies, the structure of the mouse knee is not ideal for studying meniscus pathology. Meniscus injury and repair models in addition to models to explore osteoarthritis development may be better studied in larger animal models. A comparison between human, sheep, and rabbit meniscus found that human and sheep menisci were similar and significantly different from the rabbit meniscus in terms of cellularity and vascular penetration\textsuperscript{101}. A recent comparison of three sheep models for knee osteoarthritis found meniscus body transection and capsular release as viable models for experimental osteoarthritis\textsuperscript{127}. Meniscus injury and repair in the sheep model likely provides an effective model for intervention since meniscus injuries lead to the development of osteoarthritis\textsuperscript{128}. Larger animal models such as the sheep offer less technical difficulty for creating and repairing
specific types of meniscus defects and subsequently analyzing synovial fluid and biomarkers. Larger animal models may then explore a wider range of repair and therapeutic techniques for meniscus injury and degeneration.

**Therapeutic intervention for meniscus injury**

Our results indicate that degenerative and osteoarthritic menisci respond to pro-inflammatory factors with increased production of matrix-degrading enzymes, cytokines and chemokines. Furthermore, when we treated healthy meniscus cells with pro-inflammatory factors associated with joint injury and disease, we elicited responses consistent with osteoarthritis pathogenesis and the progressive cycle of inflammatory tissue destruction. Many anti-cytokine therapies are being tested in osteoarthritis with varying degrees of success to date. The cause for variable results is likely multifactorial, as emphasized by a recent osteoarthritis consortium, but may be partially attributed to the timing of the intervention in the disease process. Since the meniscus is involved early in osteoarthritis and changes in the meniscus parallel those changes observed in articular cartilage early in the disease progression, anti-cytokine therapy may be an effective early intervention in the meniscus disease process.

The most dynamic responses in meniscus catabolic expression occurred with IL-1β and FnF treatment followed by IL-6 treatment. Since current therapeutics exist that inhibit the effects of IL-1, use of these inhibitors would offer the most immediate opportunity for intervention. A trial of individual inhibition IL-1 and TNF-α in porcine meniscus explants significantly improved repair strength, cell migration, and tissue formation at the site for full thickness meniscus defect in the explant. Another porcine meniscus explant study demonstrated that catabolic gene expression in response to compressive strains was reduced following inhibition of IL-1. The paradigm may be expanded to include anti-IL-6 and anti-IL-7 therapies. Since IL-7 is also implicated in osteoarthritis pathogenesis, reduction of intraarticular IL-7 levels may
offer clinical benefit. Reducing the catabolic activity of injured meniscus, especially that of MMPs, would improve the meniscus repair\textsuperscript{112}.

The catabolic response of the meniscus to FnF stimulation was very robust, but inhibiting the deleterious effects of FnFs on the meniscus is difficult to accomplish secondary to its multiple isoforms with varying degrees of biological activity\textsuperscript{153-159}. One strategy to block catabolic effects of FnF may be found through hyaluronic acid, which reduced FnF-mediated degradation of proteoglycan content\textsuperscript{160-163}. The clinical effectiveness of hyaluronic acid for osteoarthritis is variable\textsuperscript{164,165}, but it may have relevant biological activity for the meniscus\textsuperscript{166-170}. In a rabbit meniscus injury model, hyaluronic injected joints had a slightly more favorable repair than saline injected joints\textsuperscript{168}. Hyaluronic acid was demonstrated to increase matrix deposition of chondroitin sulfate and collagen type II in adult chondrocytes which may positively influence healing of the avascular region of the meniscus\textsuperscript{166}. Hyaluronic acid was also implicated in lung tissue repair through modulation of the NF-κB pathway in response to injury\textsuperscript{171}, which may be involved in the reported healing of meniscus. Additional studies are warranted to examine the basic biologic and clinical effects of hyaluronic acid on the meniscus.

**Conclusion**

The understanding of the biologic activity of the meniscus in osteoarthritis is in its infancy. Our research has ultimately produced divergent paths of future meniscus exploration. We have established that the meniscus is biologically active in response to pro-inflammatory stimulation in a manner that is complementary to that of articular cartilage. Catabolic responses of the meniscus provide an opportunity for therapeutic intervention to improve meniscus repair and possibly prevent or at least attenuate the development of osteoarthritis. We have additionally demonstrated that the HIF pathway did not respond as expected in the development of osteoarthritic changes in the meniscus, but the HIF pathway may play an important role in both protective and reparative
responses in the meniscus and may influence cell phenotype through differential expression of extracellular matrix proteins. This line of research has important implications for meniscus healing and protection under catabolic stress. So rather than defining the involvement of meniscus alterations in osteoarthritis development, we have established a firm foundation for continued basic science research and clinical opportunities with promising insights into treatment of the spectrum of meniscus disorders in an effort to reduce the burden of osteoarthritis.
References


57. Loeser RF, Sadiev S, Tan L, Goldring MB. Integrin expression by primary and immortalized human chondrocytes: Evidence of a differential role for alpha1beta1 and alpha2beta1 integrins in
mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 2000; 8(2): 96-105.


69. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) 2009; 2497-106.


72. Lafont JE, Talma S, Murphy CL. Hypoxia-inducible factor 2α is essential for hypoxic induction of the human articular chondrocyte phenotype. Arthritis & Rheumatism 2007; 56(10): 3297-3306.


95. Sartori-Cintra AR, Mara CS, Argolo DL, Coimbra IB. Regulation of hypoxia-inducible factor-1alpha (hif-1alpha) expression by interleukin-1beta (il-1 beta), insulin-like growth factors i (igf-i) and ii (igf-ii) in human osteoarthritic chondrocytes. Clinics (Sao Paulo) 2012; 67(1): 35-40.


99. Duval E, Leclercq S, Elissalde J-M, Demoor M, Galéra P, Boumédiene K. Hypoxia-inducible factor 1α inhibits the fibroblast-like markers type i and type iii collagen during hypoxia-induced chondrocyte redifferentiation: Hypoxia not only induces type ii collagen and aggrecan, but it also inhibits type i and type iii collagen in the hypoxia-inducible factor 1α-dependent redifferentiation of chondrocytes. Arthritis & Rheumatism 2009; 60(10): 3038-3048.


Table 1: Review of HIF studies pertaining to joint tissues.

<table>
<thead>
<tr>
<th>Author</th>
<th>Cell Type</th>
<th>HIF Genes Evaluated</th>
<th>Results (HIF gene followed by targets)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu et al, 2003&lt;sup&gt;34&lt;/sup&gt;</td>
<td>786-O WT-8 cells, HEK293 cells, HUVEC, HMVEC-L, HepG2, Hep3B</td>
<td>HIF-1α, HIF-2α</td>
<td>HIF-1α→↑multiple glycolytic genes (LDHA, PGM-1, ADPR, PKM, PGK-1, ALDA)</td>
<td>Normoxic and hypoxic studies with multiple cell lines</td>
</tr>
<tr>
<td>Pfander et al, 2003&lt;sup&gt;25&lt;/sup&gt;</td>
<td>Mouse epiphyseal chondrocytes</td>
<td>HIF-1α</td>
<td>HIF-1α→↑Glut-1, PKG-1, VEGF HIF-1α mediates glycolytic rate HIF-1α null cells decreased aggrecan and collagen type II expression when exposed to hypoxia</td>
<td>Normoxic and hypoxic studies</td>
</tr>
<tr>
<td>Comimbra et al, 2004&lt;sup&gt;93&lt;/sup&gt;</td>
<td>Human normal and osteoarthritic chondrocytes</td>
<td>HIF-1α, HIF-2α</td>
<td>HIF-1α and HIF-2α expressed in normal and osteoarthritic cells in normoxia and hypoxia TNF-α treatment stabilized HIF-1α</td>
<td>Normoxic and hypoxic studies. Inhibition of NF-κB and p38 blocked TNF-α stimulation of HIF-1α</td>
</tr>
<tr>
<td>Lin et al, 2004&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Human normal chondrocytes, chondrosarcoma cell lines</td>
<td>HIF-1α</td>
<td>HIF-1α→↑VEGF</td>
<td>Absent staining for HIF-1α protein in normal cartilage.</td>
</tr>
<tr>
<td>Brucker et al, 2005&lt;sup&gt;76&lt;/sup&gt;</td>
<td>Bovine chondrocytes and explants</td>
<td>HIF-1α</td>
<td>Stabilization and hypoxic induction of HIF-1α increased proteoglycan synthesis</td>
<td>Normoxic and hypoxic studies HIF-1α active in bovine cartilage</td>
</tr>
<tr>
<td>Yudoh et al, 2005&lt;sup&gt;92&lt;/sup&gt;</td>
<td>Human osteoarthritis chondrocytes</td>
<td>HIF-1α</td>
<td>↑HIF-1α staining in damaged human cartilage IL-1β and H₂O₂→↑HIF-1α in normoxia hypoxia</td>
<td>Normoxic and hypoxic studies IL-1β acted through MAPK family</td>
</tr>
<tr>
<td>Adesida et al, 2007&lt;sup&gt;86&lt;/sup&gt;</td>
<td>Human osteoarthritic meniscus cells</td>
<td>HIF-1α</td>
<td>Inner meniscus cells: ↑HIF-1α vs. outer Outer meniscus cells Hypoxia→↑Sox9, Col2α1, Col1α1 ↑HIF-1α in normoxia, hypoxia</td>
<td>Normoxic and hypoxic studies High variability with results, n=3 donors</td>
</tr>
<tr>
<td>Lafont et al, 2007&lt;sup&gt;72&lt;/sup&gt;</td>
<td>Human normal chondrocytes, primary and passaged</td>
<td>HIF-1α, HIF-2α</td>
<td>HIF-1α→↑Glut-1 HIF-2α→↑Sox9, Col2α1, Col9α1, VEGF</td>
<td>Normoxic and hypoxic studies</td>
</tr>
<tr>
<td>Lafont et al, 2008&lt;sup&gt;71&lt;/sup&gt;</td>
<td>Human normal chondrocytes</td>
<td>HIF-1α, HIF-2α</td>
<td>HIF-2α→↑Sox9, Gdf10, Chm-1 HIF-2α→↑Mig, Inhba (Sox9 independent)</td>
<td>Normoxic and hypoxic studies, extensive microarray studies</td>
</tr>
<tr>
<td>Duval et al, 2009&lt;sup&gt;99&lt;/sup&gt;</td>
<td>Human osteoarthritic chondrocytes, both primary and passaged</td>
<td>HIF-1α</td>
<td>HIF-1α→↑Sox9, Col2α1 and ↓Col1α1, Col1α2, Col3α1 in hypoxia</td>
<td>Hypoxic studies</td>
</tr>
<tr>
<td>Saito et al, 2010&lt;sup&gt;98&lt;/sup&gt;</td>
<td>Mouse chondrocytes Human ATDC5 cells</td>
<td>HIF-1α, HIF-2α</td>
<td>HIF-2α→directly ↑Col10α1, MMP13, VEGF HIF-2α→likely ↑MMP3, MMP9, RUNX2, IHH, PTH1R HIF-2α→↑Adams4, Adams5 TNF-α, IL-1β→↑HIF-2α expression</td>
<td>Extensive study of mouse responses and human ATDC5 cell lines. Only HIF-2α expression evaluated</td>
</tr>
<tr>
<td>Reference</td>
<td>Cells/Conditions</td>
<td>HIFs/Signaling Pathways</td>
<td>Notes</td>
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<tr>
<td><strong>Yang et al, 2010</strong></td>
<td>Mouse, rabbit chondrocytes; human osteoarthritic chondrocytes</td>
<td>HIF-2α → directly ↑MMP1, MMP3, MMP9, MMP12, MMP13, Adamts4, NOS2, PTGGS2; HIF-2α ↔ MMP2, MMP14, MMP15, Adamts5</td>
<td>Extensive study in mice and rabbit with evaluation of human osteoarthritic chondrocytes</td>
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<tr>
<td><strong>Thoms et al, 2010</strong></td>
<td>Human normal chondrocytes</td>
<td>HIF-2α → ↑Sox9</td>
<td>Normoxic and hypoxia studies focusing on for stability of HIF-2α and extracellular matrix production</td>
<td></td>
</tr>
<tr>
<td><strong>Ryu et al, 2011</strong></td>
<td>Mouse chondrocytes, human osteoarthritic chondrocytes</td>
<td>HIF-2α ↔ ↑IL-6, MMP3, MMP13</td>
<td>IL-6 is both a target gene and inducer of HIF-2α and promotes cartilage destruction.</td>
<td></td>
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<tr>
<td><strong>Ryu et al, 2012</strong></td>
<td>Mouse chondrocytes, human osteoarthritic chondrocytes</td>
<td>Anti-Fas (CD95) treatment ↑HIF-2α expression and ↑apoptosis; Mouse over expression of HIF-2α ↑apoptosis</td>
<td>Normoxic Studies</td>
<td></td>
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<tr>
<td><strong>Sartori-Cintra et al, 2012</strong></td>
<td>Human osteoarthritic chondrocytes</td>
<td>IL-1β → ↑HIF-1α protein but ↔HIF-1α gene IGF-1 → ↑HIF-1α protein and gene IGF-2 ↔ ↑HIF-1α protein and gene</td>
<td>Normoxic studies</td>
<td></td>
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<tr>
<td><strong>Adesida et al, 2013</strong></td>
<td>Human osteoarthritic meniscus cells expanded with FGF2</td>
<td>Hypoxia ↓ collagen type II versus type I expression when cultured on 3d construct; Hypoxia ↑ aggrecan versus versican expression</td>
<td>Normoxic and hypoxic studies. Evaluated effects of monolayer versus 3d culture, but high variability in results</td>
<td></td>
</tr>
<tr>
<td><strong>Gilkes et al, 2013</strong></td>
<td>Human fibroblasts</td>
<td>HIF-1α → ↑extracellular matrix deposition, alignment and directional fibroblastic cell motility via HIF-1α depended mechanism</td>
<td>Normoxic and hypoxic studies</td>
<td></td>
</tr>
<tr>
<td><strong>Thoms et al, 2013</strong></td>
<td>Human normal chondrocytes, pig chondrocytes, mouse chondrocytes</td>
<td>HIF-1α, HIF-2α绑定 to and expression of Sox9 in human and pig (not mouse); HIF-1α ↔↑MMP13; HIF-2α → ↑HIF-1α in normoxia and hypoxia and ↓HIF-2α in hypoxia</td>
<td>Normoxic and hypoxic studies in multiple species. Hypoxia protected spontaneous and induced degradation of human cartilage.</td>
<td></td>
</tr>
</tbody>
</table>
Curriculum Vitae

AUSTIN V. STONE, M.D.
Department of Orthopaedic Surgery, Medical Center Blvd, Winston-Salem, NC 27157-1070
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EDUCATION

Resident, Physician Scientist Training Program, Department of Orthopaedic Surgery 2017 (anticipated)
Wake Forest University School of Medicine, Winston-Salem, NC

Ph. D. Candidate, Molecular Medicine and Translational Sciences 2013 (anticipated)
Wake Forest University Graduate School of Arts and Sciences, Winston-Salem, NC

University of Cincinnati College of Medicine, Cincinnati, OH
M.D. 2010

Wake Forest University, Winston-Salem, NC
B.S. Honors in Biology 2005
Areas of Concentration: Protein Biochemistry, Basic Science Research
Minor: Chemistry
Honors Thesis: “Strategies for purification of the NPA Binding Protein from Arabinopsis thaliana using an ANPA affinity resin.”

AWARDS

First Place, Division of Surgical Sciences Resident Research Day November 2012
First Place, Dept. of Orthopaedic Surgery Resident Research Day April 2012
Runner-up, Translational Science Award, WFU Graduate School of Arts and Sciences March 2012
First place, Orthopaedic Research Society/Orthopaedic Research and Education October 2011
Foundation Research Atlantic Coast Symposium
First place, North Carolina Orthopaedic Association Resident Presentation October 2011
First place, Clinical Orthopaedic Society Resident Presentation September 2011
Runner-up, Translational Science Award, WFU Graduate School of Arts and Sciences September 2010
American Orthopaedic Society for Sports Medicine Young Investigator Travel Award June 2007
Golden Scalpel Award, Dept. of Anatomy, UCCOM May 2005
Graduation with Honors in Biology
Wake Forest University Senior Colloquium, Speaker (professor nominated and selected) April 2005
Elton C. Cocke Scholarship Award, Wake Forest University Department of Biology March 2005
First Place for Oral Presentation, Annual Meeting, North Carolina Academy of Sciences March 2005
Wake Forest University Dean’s List 2002 – 2005
Finalist, NASA/Boeing International Space Station Design Competition 2001

GRANTS

American Orthopaedic Society for Sports Medicine
March 2011
Young Investigator Grant, Principle Investigator Molecular Mechanisms in Meniscus Injury that Contribute to Subsequent Osteoarthritis.

Orthopaedic Research & Education Foundation (OREF)
February 2011
Resident Clinician Scientist Training Grant, Principle Investigator The Role of HIF-2α and NF-κB in meniscus degeneration.

Orthopaedic Research & Education Foundation (OREF): Medical Student Summer Orthopaedic Research Fellowship, Co-PI Is Osteochondritis dissecans a growth plate disorder?
March 2007

University Orthopaedic Research & Education Foundation Faculty Research Grant Is Osteochondritis dissecans a growth arrest of the secondary ossification center?
October 2007
Co-investigator

Wake Forest University Research Fellowship
Purification of the NPA Binding Protein in Arabinopsis thaliana
August 2004
Wake Forest University Research Fellowship

Localisation of the NPA Binding Protein in Arabidopsis thaliana

May 2004

CERTIFICATION AND LICENSURE

North Carolina State Board of Medicine Training License 2012 – present
HIPPA/OSHA Certification 2006 – present
CITI – Course in The Protection of Human Research Subjects 2005 – present
College Reading and Learning Association Tutor Accreditation 2005

RESEARCH EXPERIENCE

Cincinnati Children’s Hospital Medical Center, Cincinnati, OH
Medical Student Research Fellow, Department of Orthopaedic Surgery 2007 - 2010

Wake Forest University School of Medicine, Winston-Salem, NC
Research Assistant 2005 – 2006
Department of Orthopaedic Surgery

Wake Forest University, Winston-Salem, NC
Student Research Fellow, Department of Biology 2003 – 2005
Learned experimental design, developed strong background in biochemical, molecular, and bioanalytical techniques.

Wake Forest University School of Medicine, Winston-Salem, NC
Research Intern 2003 – 2005
Department of Orthopaedic Surgery

PUBLICATIONS AND PAPERS

MANUSCRIPTS


Stone AV. Strategies for purification of the NPA Binding Protein from Arabidopsis thaliana using an ANPA affinity resin. [Senior Honors Thesis] 2005

BOOK CHAPTERS


ABSTRACTS AND PRESENTATIONS

ORAL PRESENTATIONS

National/International

A potential biological mechanism for meniscus involvement in osteoarthritis. Annual Meeting of the Clinical Orthopaedic Society September 2011

Early results following bovine xenograft placement for pediatric Cotton and Evan’s osteotomies. Annual Meeting of the Clinical Orthopaedic Society September 2011
Dose and volume dependent response to intramuscular injection of botulinum neurotoxin-A optimizes muscle force decrement in mice. Residents and Fellows Conference at Annual Meeting of American Society for Surgery of the Hand  

Repetitive Stresses Generate Juvenile Osteochondritis Dissecans-Like Changes in Immature Animals. Annual meeting of the American Academy of Orthopaedic Surgeons  

Local/Regional Meetings  
A potential biological mechanism for meniscus involvement in osteoarthritis.  
Orthopaedic Research Society/Orthopaedic Research and Education Foundation Atlantic Coast Research Symposium  

Human osteoarthritic and degenerative monkey menisci have differential increases of matrix metalloproteases compared to both osteoarthritic chondrocytes and healthy monkey menisci. Annual Meeting of the Eastern Orthopaedic Association.  

A potential biological mechanism for meniscus involvement in osteoarthritis.  
Annual Meeting of the North Carolina Orthopaedic Society  

Repetitive Stresses Generate Osteochondral Lesions in Immature Animals.  
Orthopaedic Research Society Atlantic Coast Conference  

Differential expression of a potential biological mechanism for meniscus involvement in osteoarthritis.  
Orthopaedic Research Society Atlantic Coast Conference  

A potential biological role for the meniscus in osteoarthritis.  
Graduate Student & Post-Doctoral Research Day, WFU Grad. Schol of Arts and Sciences  

A potential biological role for the meniscus in osteoarthritis.  
Surgical Sciences Research Day, Wake Forest School of Medicine  

Differential matrix metalloprotease expression in human osteoarthritic meniscal cells stimulated with inflammatory cytokines.  
Graduate Student & Post Doctoral Research Day, WFU Grad. Schol of Arts and Sciences  

ABSTRACTS  


Koman LA, Plate JF, Mannava S, Stone AV, Jinnah RH, Smith TL. Addressing the shortage of academic orthopaedic surgeons: Evaluation of an Innovative seven-year physician scientist residency training program. AOA 2012
Koman LA, Plate JF, Mannava S, Stone AV, Jinnah RH, Smith TL. Addressing the shortage of academic orthopaedic surgeons: Evaluation of an Innovative seven-year physician scientist residency training program. AAOS 2012

Stone AV, Loeser RF, Long DL, Ferguson CM. A potential biological mechanism for meniscus involvement in osteoarthritis. ORS 2012

Stone AV, Long DL, Loeser RF, Ferguson CM. Human osteoarthritic and degenerative monkey menisci have differential increases of matrix metalloproteases compared to both osteoarthritic chondrocytes and healthy monkey menisci. EOA 2011

Koman LA, Plate JF, Mannava S, Stone AV, Jinnah RH, Smith TL. Addressing the shortage of academic orthopaedic surgeons: Evaluation of an Innovative seven-year physician scientist residency training program. EOA 2011

Stone AV, Long DL, Loeser RF, Ferguson CM. A potential mechanism for meniscus involvement in degenerative joint disease. NCOA 2011

Koman LA, Plate JF, Mannava S, Stone AV, Jinnah RH, Smith TL. Addressing the shortage of academic orthopaedic surgeons: Evaluation of an Innovative seven-year physician scientist residency training program. NCOA 2011

Stone AV, Plate JF, Gyr BM, Scott AT. Early results following bovine xenograft placement for pediatric Cotton and Evan’s osteotomies. COS 2011

Stone AV, Long DL, Loeser RF, Ferguson CM. A potential biological mechanism for meniscus involvement in osteoarthritis. COS 2011

Plate JF, Stone AV, Seyler TM, Marker DR, Akbar M, Mont MA. Accuracy of diagnosing periprosthetic infections by both cultures and frozen sections. COS 2011

Plate JF, Stone AV, Seyler TM, Akbar M, Mont MA. Comparison of dislocation rates in patients with large diameter metal-on-metal and small diameter metal-on-polyethylene bearings in total hip arthroplasty. COS 2011


Ma J, Stone AV, Smith BP, Callahan M, Garrett J, Smith TL, Koman LA. Dose and volume dependent response to intramuscular injection of botulinum neurotoxin-A optimizes muscle force decrement in mice. ASSH 2011


DEPARTMENTAL PREsentATIONs

Beyond the Biomechanics: The Meniscus in Osteoarthritis Pathogenesis 
Dept. of Molecular Medicine & Translational Sciences, WFSM November 2012

A Biologic Role for the Meniscus in Osteoarthritis Pathogenesis 
Dept. of Orthopaedic Surgery, WFSM April 2012

A Biologic Mechanism for the Meniscus in Osteoarthritis Development 
Dept. of Molecular Medicine & Translational Sciences, WFSM April 2012

Osteoarthritic and Degenerative Changes in the Meniscus 
Resident Research Day, Department of Orthopaedic Surgery, WFSM April 2011
Differential matrix metalloprotease expression in human osteoarthritic meniscus cells versus osteoarthritic chondrocytes stimulated with inflammatory cytokines
Wake Forest Institute for Regenerative Medicine

March 2011

Elucidating the Role of HIF-2α in Osteoarthritis
Dept. of Molecular Medicine & Translational Sciences, WFSM

November 2010

Medical Issues in the Orthopaedic Patient
Internal Medicine Conference: The Jewish Hospital, Cincinnati, Dept. of Internal Medicine

February 2010

Scaphoid Nonunion Advanced Collapse and Scapholunate Advanced Collapse:
Current diagnosis and treatment options.
Univ. of Cincinnati College of Medicine, Dept. of Orthopaedic Surgery

May 2009

Renal Trauma: Assessing indications for exploration.
University of Cincinnati Trauma Division, Dept. of Surgery.

November 2008

Is juvenile osteochondritis dissecans a result of injury to the secondary ossification center?

July 2007

TEACHING EXPERIENCE
Wake Forest University, Research Mentor, Undergraduate Research 2011 – 2012

Wake Forest School of Medicine, Winston-Salem, NC
Mentor, Medical Student Research Proposal and Project 2011

Wake Forest School of Medicine, Winston-Salem, NC
Clinical Volunteer Faculty Facilitator 2011

Life and Physical Sciences, Wake Forest University, Winston-Salem, NC
College Tutor, CRLA Accredited 2002 – 2005

Brunson Elementary, Winston-Salem, NC
Science Educator, 5th grade, American Chemical Society, Volunteer 2004 – 2005

Mount Washington Presbyterian Church, Cincinnati, OH

OTHER POSITIONS AND EMPLOYMENT
Knowledge2Work, Winston-Salem, NC
Chief Information Officer (CIO) 2003 – 2004

Managed Information Technologies group; lead initiative for integration of cutting edge technologies and infrastructure expansion.

Newsarama.com, Winston-Salem, NC
Consultant 2004

Advanced design and optimized page processing for enhanced usability.

PsychTemps, Inc., Cincinnati, OH
Consultant and Developer 2001 – 2003

Enterprise application design and development; internet presence and e-commerce consulting with internal process optimization.

PROFESSIONAL DEVELOPMENT
Wake Forest Baptist Health Arthroscopy and Trauma Labs
OREF/ORS Grant Writing Workshop
Southeastern Fracture Symposium
Trauma and Reconstruction Laboratory and Course
Wake Forest Baptist Health Orthopaedic Surgery Knee Arthroscopy Lab, Coordinator
American Orthopaedic Society for Sports Medicine Clinical Outcomes Research
Wake Forest Department of Orthopaedic Surgery Trauma Call Course
OREF/ORS Presentation Skills Workshop
American Orthopaedic Society for Sports Medicine Post-Joint Injury
Osteoarthritis Conference

Quarterly
February 2012
January 2012
October 2011
August 2011
July 2011
June 2011
December 2010
December 2010
Microsurgery Course, Dept of Orthopaedic Surgery, WFU School of Medicine 2005 – 2006
Research intern, Dept of Orthopaedic Surgery, Wake Forest University School of Medicine 2003 – 2005
LEAD – Leadership Training and Development Program Graduate 2002

COMMUNITY SERVICE
Wake Forest University Alumni Career Assistance Program, Volunteer Advisor 2009 – present
Church Musician, Mount Washington Presbyterian Church, Volunteer 2006 – 2010
Anthem Blue Cross/Blue Shield Cycling Team, Team Member, Cyclist 2006 – 2008
National MS Society MS Bike Tour, Cyclist/Fundraiser 2004 – 2006
Volunteer, Operating Room, Wake Forest University Baptist Medical Center 2002 – 2003

PROFESSIONAL MEMBERSHIPS
Orthopaedic Research Society, Associate Member
Graduate Student Association, MD liaison for Mol. Med. Trans. Sci.

EXTRACURRICULARS
Cycling, Culinary Endeavors, Musician, General Contractor