

**THE REGULATION OF PRG4
VIA F-ACTIN REORGANIZATION
IN NATIVE SUPERFICIAL ZONE CHONDROCYTES**

by

Sofia Gonzalez-Nolde

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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ABSTRACT

Proteoglycan 4 (Prg4) is a critical regulator of cartilage homeostasis; it is a surface lubricant and suppresses inflammatory processes. Studies show that Prg4 is chondroprotective and can prevent the onset of Osteoarthritis (OA). *In vitro* studies have demonstrated that the actin cytoskeleton is a strong regulator of Prg4. While these *in vitro* studies may suggest that targeting the actin cytoskeleton in superficial zone chondrocytes (SZCs) may provide a novel therapeutic approach against OA, it is not known whether Prg4 is regulated by filamentous (F-)actin reorganization in native cartilage environment. Furthermore, it is not certain if, and the mechanisms by which F-actin reorganizes in native tissue. To gain an understanding of F-actin regulation in native cartilage, we developed a native tissue model that mimics aspects of mRNA level gene changes in OA. This model involves isolating mouse femoral head and culturing femoral heads in a serum-free media. In addition to a reduction in matrix, and increases in proteases, we determine that culturing femoral heads leads to a reduction in Prg4 mRNA levels. To elucidate the regulation of Prg4 by F-actin in native SZCs, we developed a novel whole mount imaging approach which allows for high spatial resolution visualization of F-actin organization in native SZCs. Using this approach, we reveal that F-actin reorganizes in native SZCs with an increase in the proportion of F-actin to globular (G-)actin by *ex vivo* culture of femoral heads. Treatment with TGF- β promotes F-actin polymerization and sustains F/G-actin in culture while stimulating Prg4 expression. The regulator of F-actin in elongated cells, Tropomyosin3.1 (Tpm3.1), was found to be critical for this induction. Inhibition or

knockout of Tpm3.1 prevented the induction of Prg4 by TGF- β . This study is critical in understanding how F-actin regulates Prg4 in native SZCs. Elucidating the regulation of Prg4 via F-actin in native tissue will allow for the uncovering of potential molecular targets against OA.

Chapter 1

FROM FORM TO FUNCTION: TARGETING CELLULAR ARCHITECTURE VIA THE ACTIN CYTOSKELETON TO REGULATE PRG4

1.1 Abstract

The superficial zone of articular cartilage is a target of interest for preventing the onset of Osteoarthritis (OA) because it is the first to degrade. The superficial zone (SZ) is responsible for protecting the tissue against shearing forces and contributing to joint lubrication through the production of proteoglycan-4 (Prg4). Prg4 has a chondroprotective effect and there is evidence it is a molecule that can prevent the onset of OA via overexpression. *In vitro* studies show regulation via the actin cytoskeleton and there remain many growth factors that modulate Prg4 while affecting the cytoskeleton. We review the regulation of Prg4 via the actin cytoskeleton and pose unanswered questions about the actin in the SZ as a molecular target for OA prevention.

Key Words: Osteoarthritis, Superficial zone chondrocytes, Actin Cytoskeleton, Proteoglycan-4 (Prg4), Tropomyosins

1.2 Superficial Zone Chondrocytes: A Target for Preventing Osteoarthritis

The superficial zone (SZ), also called the tangential zone, is the thinnest layer of articular cartilage that makes up the first 10-20% of cartilage thickness¹ (Figure 1.1). The sole cell type of the superficial zone is superficial zone chondrocytes (SZCs) that produce a high amount of collagen, but a low production of proteoglycans relative

to deeper zones¹. SZ collagen fibers are aligned parallel to the articular surface to aid in the tensile strength of articular cartilage¹. OA initiates in the SZ^{2,3}. The SZ experiences changes such as cellular disorganization, apoptosis, fibrillation, and SZC clustering^{4,5}. Once this zone begins degrading, deeper zones subsequently degrade; changes in the superficial zone precede deep tissue remodeling⁶. Ultimately, the dysregulation of SZCs is a crucial first step in OA pathogenesis⁷. Therefore, SZCs may provide a target to protect against OA.

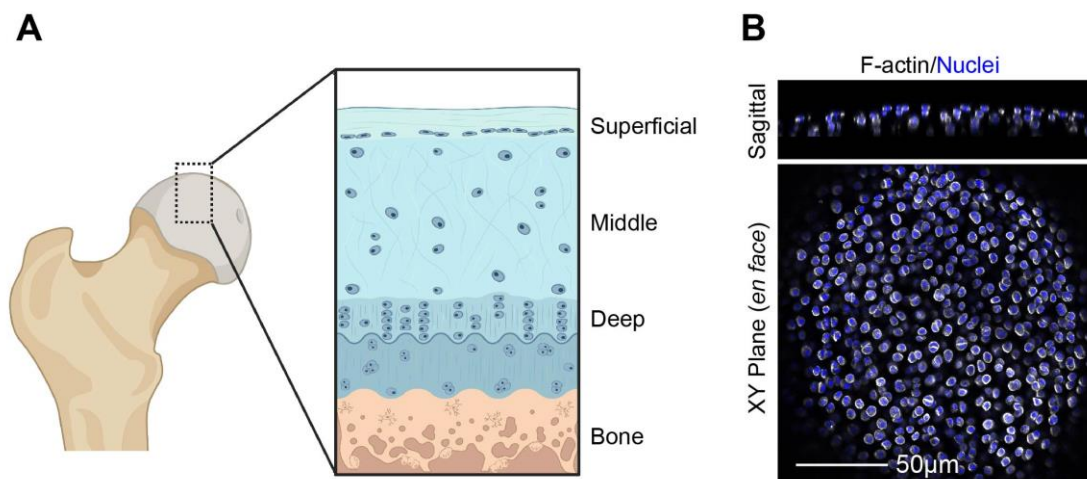


Figure 1.1 Cellular zonal organization of articular cartilage. [A] Chondrocyte cells are situated upon calcified bone in a superficial (SZ), middle (MZ), and deep zone (DZ) arrangement. [B] SZCs are small and elongated in a sagittal view and are round with cortical F-actin arrangement from *en face* view.

Lubricin or proteoglycan-4 (Prg4), homologous with megakaryocyte stimulating factor, is a mucinous glycoprotein present at the articular surface that functions as a boundary lubricant and is essential for the maintenance of cartilage homeostasis⁸. While also expressed in the synovium, Prg4 in articular cartilage is exclusively expressed, produced, and secreted by SZCs⁹. Prg4 has been regarded as an

important friction reducing component in joint surfaces and participates in lowering the coefficient of friction between joint surfaces to the order of ~0.01 or less¹⁰. It works synergistically with hyaluronic acid to produce a lubricating effect^{11,12}. The lubrication prevents matrix degradation and is cytoprotective⁸; in the absence of Prg4, an increase of friction causes mitochondrial dysfunction in chondrocytes¹³. Prg4 plays an anti-inflammatory role by binding to toll-like receptors 2, 4, and 5¹⁴ thereby reducing inflammation in the synovium¹⁵ through the inhibition of matrix metalloproteinase activity¹⁶⁻¹⁸.

Prg4 is essential for joint health. In humans, loss-of-function mutations to the Prg4 gene causes Camptodactyly-Arthropathy-Coxa Vara-Pericarditis Syndrome which is characterized by joint abnormalities including childhood onset OA¹⁹. Additionally, it is postulated that the loss of Prg4 expression and secretion contributes to the onset of OA. In animal models, Prg4 is downregulated in early OA²⁰⁻²² resulting in an increased coefficient of friction in the synovial fluid and cell death. In mice, knock out of Prg4 leads to joint failure including an increase in swelling, calcification, and cartilage degeneration²³. Furthermore, the chondroprotective effect of Prg4 has been exemplified by supplementing mouse joints with Prg4 to prevent OA^{10,24-26}. In a similar fashion, Prg4 overexpression protects against post-traumatic and age-related OA by inhibiting cartilage catabolism and hypertrophy²⁷.

Due to the critical function Prg4 plays in establishing healthy, functional joints and the known reduction of Prg4 in early OA, understanding the regulation of Prg4 is a great area of interest. Unlocking the molecular mechanisms regulating Prg4 may lead to novel therapeutic targets against OA. Previous studies have indicated several molecular pathways involved in regulating Prg4 including transforming growth factor

beta (TGF- β), Wnt signaling, epidermal growth factor receptor (EGFR), transient receptor potential vanilloid channel (TRPV), Prostaglandin E2 (PGE2), and parathyroid hormone-related peptide (PTHrP). These signaling pathways have been discussed in previous literature reviews^{22,28-30}. In addition to these signaling pathways, a connection between SZC form and function has been demonstrated. SZC shape and cytoskeleton regulate SZC phenotype, including Prg4 expression³¹⁻³³. The actin cytoskeleton may be a critical node in signal transduction of these established pathways and provide a druggable target in regulating downstream Prg4 expression in SZCs^{34,35}. As we review here, Prg4 is mediated by actin polymerization status, chemical and mechanical stimuli; we suggest the latter may depend non-canonically on the actin cytoskeleton. In this review we aim to: (i) synthesize established Prg4 pathways via the actin cytoskeleton (ii) speculate unknown connections of Prg4 regulatory elements to the actin cytoskeleton.

1.3 Cytoskeleton Components of SZCs

Depth-dependent chondrocyte shape and function is reliant on the three-dimensional (3D) cytoskeletal network composed of microtubules (MTs), vimentin intermediate filaments (IFs), and F-actin microfilaments. The gross morphology of the cytoskeleton network in chondrocytes, includes dense F-actin staining with punctate and cortically arranged filaments just below the chondrocyte cell membrane^{36,37}. MTs are found distributed evenly in the cytoplasm, forming a mesh-like structure. IFs are also found throughout the cytoplasm in a tight mesh and connect from the plasma membrane to the nuclear membrane. Depth-dependent cell morphology of chondrocytes is coupled with unique cytoskeletal elements in articular cartilage; as compared to deep zone chondrocytes which are oblong and sparsely distributed, SZCs

are thin, relatively densely packed and horizontally arranged parallel to the cartilage surface. IFs and MTs are visibly more abundant in SZCs compared to deeper zones while F-actin microfilaments were found to be more uniformly distributed in the periphery of joints. However, in load bearing regions of joints, chondrocytes show less depth-dependent, more uniform cytoskeletal arrangement of IFs and MTs connections compared to regions in the periphery³⁶. This suggests that a specific cytoskeletal arrangement is critical in the presence of load-bearing stress. In comparison to the cells from deeper zones, SZCs have a unique actin network organization. While microscopy analysis demonstrates that fluorescence staining for F-actin using Phalloidin is relatively uniform in SZCs and deeper chondrocytes, western blot analysis shows a larger amount of total actin in SZCs³⁶. Since phalloidin recognizes only polymerized F-actin, and western blot recognizes both soluble and insoluble protein, this indicates a larger pool of soluble G-actin in SZCs, possibly for greater remodeling capability. In isolated primary cells, it has been demonstrated that primary SZC have greater F/G-actin than DZC counterparts³⁸. RNA sequencing demonstrates differential cytoskeletal associated molecule expression in the SZC as compared to cells from deeper zones, which is linked to cell division control protein 42 homologue (Cdc42) signaling³⁹. In culture, SZCs have a greater propensity to form mature focal adhesions as compared to DZC. Therefore, it is likely that SZC F-actin networks are maintained by a unique set of signaling and actin network molecules; and that remodeling of F-actin networks is important for regulating SZC phenotype. The regulation of the SZC phenotype and Prg4 expression by IFs is unknown. However, the interference of MT and F-actin dynamics can reduce basal levels and the induction of Prg4 expression³¹.

1.4 The Actin Cytoskeleton as a Regulator of OA Phenotype and the Superficial Zone

The regulation of the chondrocyte phenotype, including that of the SZC phenotype, by cytoskeletal elements is best exemplified by the regulation via actin. F-actin reorganization is a potent regulator of chondrocyte phenotype with ability to regulate cartilage matrix, fibroblast matrix, proliferative, apoptotic, and transcriptional molecule expression^{40,41}. In OA pathogenesis, transcriptional analysis of differentially expressed genes show the most affected pathway in OA is the actin cytoskeleton^{42,43}. Notably, the change in actin polymerization is thought to trigger a phenotypic switch in chondrocytes. Studies on isolated cells demonstrate that the reorganization of cortical filamentous (F-)actin into elongated F-actin stress fibers leads to a decrease in cartilage matrix of collagen type II (Col2), aggrecan (Acan) and an increase in fibroblast matrix of collagen type I (Col1) and tenascin C (Tnc) gene expression^{41,44,45}.

In addition to the regulation of cartilage matrix expression, actin reorganization is a regulator of the SZC phenotype and Prg4 as shown *in vitro*³¹⁻³³. However, this regulation is unknown to be relevant to SZCs in their native matrix environment. In isolated cells *in vitro*, the proportion of globular (G-)actin to F-actin regulates chondrocyte gene expression through two downstream actin-associated signaling pathways, Yes-associated protein and transcriptional coactivator with PDZ binding motif (YAP/TAZ) and myocardin-related transcription factor (MRTF)⁴¹.

1.4.1 Upstream F-actin Depolymerization via Cdc42 Inhibition Reduces Prg4 in Part by MRTF

Basal levels of Prg4 have been shown to depend on Cdc42 which regulates the nuclear localization of MRTF^{32,33,44}. Cdc42 activation drives Rho A activity and stimulates actin polymerization⁴⁶. The exact mechanism by which Cdc42 regulates

Prg4 expression has not been elucidated completely, however Prg4 has been shown to be in part regulated by MRTF-A. MRTF is a G-actin binding transcription factor consisting of two isoforms MRTF-A and MRTF-B. MRTF contains an RPEL (arginine-proline-glutamine-leucine consensus sequence containing) domain that has three actin binding motifs. This domain overlaps a nuclear localization signal⁴⁷. When unbound to G-actin, MRTF exists in the nucleus to affect transcription⁴⁸. In the nucleus, MRTF does not directly bind to DNA, but rather acts as a co-activator of gene expression by interacting with serum response factor (SRF). SRF binds to CC(A/T)₆GG on promoter regions of target genes. In response to actin depolymerization, a decrease in F/G-actin, MRTF binds to G-actin and is sequestered in the cytoplasm of cells. Exposure of SZC to actin depolymerization agent, latrunculin B, results in cytoplasmic localization of MRTF and a reduction in Prg4 expression levels. Molecular inhibition of Cdc42 in primary SZCs with ML414 increases cell circularity and the G/F-actin ratio, subsequently reducing nuclear localization of MRTF-A³² and reduces Prg4. Molecular inhibition of MRTF-A decreases Prg4 expression. However, a knockdown of MRTF-A with small interfering RNA (siRNA) has no effect on expression, suggesting Prg4 is regulated only in part by MRTF. The difference between these two methods is that siRNA of MRTF does not completely remove functional MRTF protein while molecular inhibition meaning some MRTF protein could reside in the nucleus. This could suggest either the remaining MRTF-A in the nucleus of SZC is enough to drive Prg4 expression, compensation by MRTF-B, or other pathways are also involved in regulating Prg4.

YAP/TAZ signaling in SZCs

Cdc42 regulates Prg4 through the nuclear localization of transcriptional cofactors Yes-associated protein (YAP) and transcriptional coactivator with PDZ binding motif (TAZ). YAP/TAZ enter the nucleus when the nuclear membrane is mechanically stretched by nuclear actin⁴⁹. Like MRTF-A, YAP/TAZ itself regulates the actin cytoskeleton and actin polymerization status regulates YAP/TAZ nuclear/cytoplasmic localization.

YAP and TAZ are structurally similar transcription factors which are implicated in cellular proliferation, differentiation, and organ growth. Canonical activation of hippo signaling inhibits YAP and TAZ activity; downstream factors are activated that phosphorylate YAP/TAZ which sequesters these factors in the cytoplasm by binding them to a 14-3-3-protein^{50,51}. Non-canonical signaling pathway exists via the F-actin cytoskeleton. Angiomotin (AMOT) regulates the subcellular localization of YAP/TAZ. When YAP/TAZ is bound to AMOT, the complex localizes to the cytoplasm of cells. AMOT is capable of binding to F-actin through a conserved actin binding domain in its N-terminus⁵². Actin polymerization is mediated by the ABPs capZ, cofilin, and gelsolin that maintain cytoplasmic YAP/TAZ; knockdown of these ABPs increases transcription of YAP/TAZ target genes⁵³. As actin polymerizes, YAP/TAZ competes with F-actin for AMOT and YAP/TAZ is released from AMOT sequestration, allowing YAP/TAZ to enter the nucleus. Inside the nucleus, YAP/TAZ binds with transcriptional co-activators to regulate gene expression notably through the interaction with the TEA-domain family of transcription factors. Prg4 is a transcriptional target of YAP/TAZ. In SZC, treatment with Latrunculin B leads to decreased nuclear localization of TAZ³³. Similarly, Prg4 expression can be inhibited

by inhibiting YAP/TAZ via verteporfin or siRNA-mediated knockdown which downregulates Prg4 in primary bovine SZCs³³.

1.5 Actin as a Critical Node in Prg4 Signaling: Do Growth Factors and Cytokines Regulate Prg4 through Actin?

The mechanistic regulation of Prg4 by extracellular signals such as growth factors/cytokines and mechanical loads have led to the identification of various signaling pathways that contribute to Prg4 expression which may intersect and converge on the F-actin cytoskeleton (Figure 1.2). Cytokines including growth factors such as transforming growth factor beta (TGF- β) superfamily and others have been documented to regulate Prg4 in either an actin dependent manner or have been shown separately to affect the cytoskeleton.

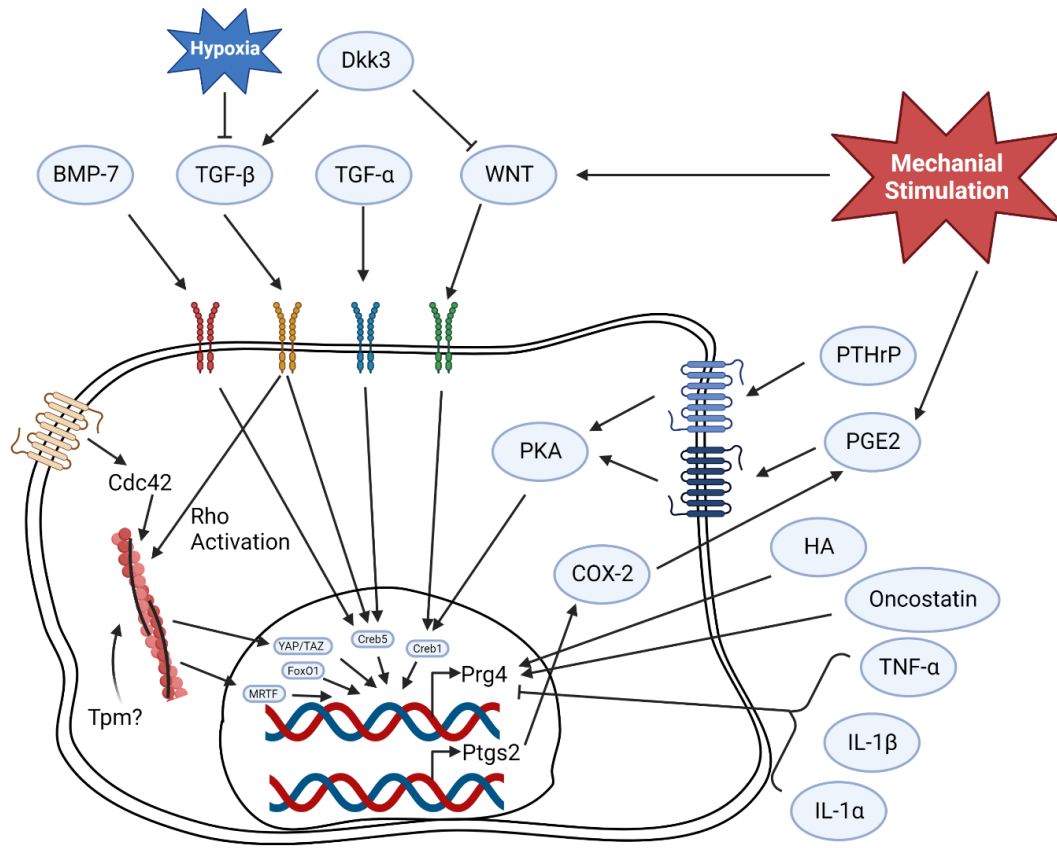


Figure 1.2 Schematic of known regulatory pathways of Prg4. Growth factor stimulation via BMP-7, TGF- β , TGF- α , and WNT stimulate Prg4 directly in a Creb dependent manner. WNT can induce Prg4 through mechanical stimulation; TGF- β also stimulates Prg4 dependent on F-actin polymerization. PTHrP and PGE2 stimulate Creb through PKA while Cox-2, a transcript of Ptg2, stimulates PGE2. Other factors HA and Oncostatin increase Prg4. Prg4 is inhibited by TNF- α , IL-1 α , and IL-1 β .

1.5.1 Disruption of F-actin Prevents TGF- β Induced Prg4 in SZCs

TGF- β induces Prg4 expression in SZCs^{31,54-57} and this induction is dependent on the actin cytoskeleton. TGF- β promotes cell spreading in primary chondrocytes, and lamellar ruffling in passaged chondrocytes, and F-actin stress fiber formation in Swiss3T3 cells, mediated by Rho GTPases^{31,58,59}. Rapid polymerization in response to TGF- β is dependent on Rho GTPases Cdc42 and RhoA, while long term stress fiber

formation is dependent on both Smad and Rho GTPase activity conjunctively⁶⁰. It remains unclear if it is either/or the rapid reorganization of actin that results in increased Prg4 transcription or the long-term stress fiber formation.

Actin depolymerization agent, cytochalasin D, leads to an overall net depolymerization of actin in chondrocytes⁴¹. Mechanistically cytochalasin D binds to barbed ends thereby preventing actin assembly/F-actin polymerization. Continued disassembly at the pointed end results in elevated pools of monomeric (G-)actin. This causes SZC treated with cytochalasin D to round and become smaller. This depolymerization also prevents Prg4 accumulation in the culture media with TGF- β treatment³¹. Similar results on Prg4 accumulation are seen with the *in vitro* actin stabilization agent, jasplakinolide³¹. It is speculated that pre-treatment of SZC with jasplakinolide prevents F-actin from reorganizing following TGF- β treatment. This is thought to inhibit the increases in F/G-actin caused by TGF- β treatment, preventing the upregulation of Prg4. Alternatively, jasplakinolide can also directly disrupt F-actin structures⁶¹. It is possible that jasplakinolide disruption of SZC F-actin leads to depolymerization of F-actin. While the actin polymerization status was not investigated in SZCs treated with jasplakinolide, in support of it disrupting SZC actin, SZCs treated with jasplakinolide are small and round. Regardless of whether jasplakinolide prevented the increase in F-actin polymerization, or caused F-actin depolymerization, the results signify that TGF- β treatment regulates Prg4 through actin mediated signaling processes.

As shown in other cell types, gene regulation by TGF- β is at least in part controlled by actin through MRTF and/or YAP/TAZ⁶²⁻⁶⁴. YAP/TAZ and MRTF protein are shown to be expressed in primary superficial zone chondrocytes. A pool

consisting of more G/F-actin leads to a decreased proportion of nuclear MRTF and YAP/TAZ in the SZC nucleus, thereby reducing Prg4^{32,33}. While TAZ expression is lower in SZC as compared to DZC, MRTF-A expression is enriched in SZC as compared to DZC³⁸. However, data thus far demonstrates that these are two pathways downstream of actin dynamics that regulate SZC gene expression. YAP/TAZ and MRTF-A signaling also intersect; MRTF is essential for TAZ expression. The two proteins can colocalize and inhibit each other's nuclear accumulation⁶⁵. Crosstalk between these factors is essential for inciting a mechanosensitive response in the context of TGF- β signaling. Disruption of actin via jasplakinolide greatly increases nuclear accumulation of MRTF-A but does not affect TAZ distribution. This suggests that actin polymerization alone is not sufficient for TAZ nuclear translocation. Thus, the induction of Prg4 by TGF- β may be mediated through MRTF and/or YAP/TAZ signaling.

1.5.1.1 Canonical Regulation of Prg4 via TGF- β May Converge on The Actin Cytoskeleton

In addition to inducing F-actin polymerization, TGF- β increases Prg4 through mechanisms independent of actin. TGF- β has been shown to regulate Prg4 through the canonical signaling via ALK5 and Smad2/3. TGF- β 3-induces Smad signaling, in conjunction with beta-catenin as (β -catenin) which is critical toward establishing cortical F-actin networks during chondrogenesis⁶⁶. Furthermore, β -catenin signaling intersects with protein degradation pathways. β -catenin can prevent the degradation of YAP/TAZ and/or MRTF which could lead to a potentiation of actin-based signaling. Wnt/ β -catenin signaling activity is elevated in the superficial zone of mouse cartilage, with knockout of β -catenin causing an acceleration of OA phenotype development and

decreased Prg4 expression. Conversely, β -catenin stabilization upregulated Prg4 expression⁶⁷. Dickkopf-3 (Dkk3) inhibits Wnt signaling and enhances TGF- β signaling and is found to be elevated in human adult OA cartilage and synovial fluid⁶⁸. That said, it is not known if Prg4 is directly affected by Dkk3.

TGF- β signaling can also activate EGFR mediated pathways. TGF- β 2 signaling through the EGFR receptor requires the binding of transcription factor Creb5 to two proximal cis-elements, E1 and E2, to increase Prg4 expression⁶⁹. E3, a distal element, drives the transcriptional activity induced by TGF- β 2. In other cell types, activation of EGFR has also been shown in other cell types to promote F-actin polymerization and nuclear MRTF localization^{70,71}. Furthermore, EGFR itself has an actin binding domain (residues 984-996) and is an actin binding protein discovered via co-sedimentation with filamentous actin⁷², although the nature of this interaction in regulating downstream gene expression is unknown.

1.5.2 BMP-7 as a Regulator of Prg4

Another member of the TGF- β superfamily, bone morphogenic protein 7 (BMP-7), works synergistically with TGF- β to increase Prg4 accumulation in which synoviocytes are more sensitive to than articular chondrocytes^{54-56,73-77}. No studies have observed BMP-7 induced changes to the cytoskeleton in SZCs, however, BMP-7 induces rapid actin cytoskeleton reorganization as potent as TGF- β in Swiss3T3 cells⁷⁸. Therefore, it is possible BMP-7 induces Prg4 accumulation in an actin dependent manner like TGF- β ; although to our knowledge, no studies investigate this. BMP-7 was originally investigated as an injectable therapy for OA because with increasing age and during OA progression, BMP-7 is decreased in expression⁷⁹⁻⁸² and through this a body of literature emerged looking into the effects of BMP-7 in

chondrocytes. However, because levels of BMP-7 have been found below detectable levels in the synovium in both patients with and without OA, we speculate the presence of BMP-7 is less important when considering the regulation of Prg4 in a pathogenesis perspective⁸³. Like BMP-7, there are other growth factors that stimulate Prg4 and simultaneously affect F-actin although these links remain speculative.

1.5.3 TGF- α as a Regulator of Prg4

TGF- α , a member of the epidermal growth factor family (EGF) increases Prg4 by binding to the EGFR in bovine cartilage explants⁸⁴. During the destabilization of the medial meniscus in mice, EGFR activity is found to be diminished⁸⁴. Furthermore, cartilage-specific *Egfr*-deficient mice develop early onset OA and have decreased surface secretion of Prg4⁸⁴. Contrary to this, functional annotation of differentially expressed genes shows enrichment of EGF/EGFR and TGF- β pathways in cartilage from OA patients⁸⁵. Granted that these pathways are upregulated in signaling in OA but generally Prg4 is reduced in OA models, this may suggest another pathway that is aberrant in the induction of Prg4, perhaps via the actin cytoskeleton. No studies have characterized the effect of TGF- α on the cytoskeleton in SZCs, however in epithelial cells, co-expression of the tetraspanin CD9 and TGF- α through EGFR activation causes a decrease in actin stress fibers through changes in RhoA and Rac1 GTPase activity⁸⁶. It also remains undetermined which downstream transcription factors EGFR activation via TGF- α increases Prg4.

1.5.4 Other Growth Factors That Regulate Prg4

Other growth factors known to enhance the accumulation of Prg4 in media include fibroblast growth factor 2 (FGF-2), insulin growth factor 1 (IGF-1), platelet-

derived growth factor (PDGF); they increase Prg4 in synoviocytes and in chondrocytes grown in monolayer. IGF-1 has been shown to increase F-actin and cell stiffening in bovine articular chondrocytes⁸⁷. The increase in F/G-actin results in downstream mediation of Prg4 via YAP/TAZ and/or MRTF remains to be delineated.

1.5.5 Prg4 Mediated by Proinflammatory Cytokines

In contrast to the growth factors, exposure of SZCs to inflammatory cytokines such as IL1- α , IL1- β , or TNF- α , significantly reduces Prg4 accumulation^{56,77,88}. Alongside this, inflammatory cytokines have been shown to change cell morphology and cytoskeleton arrangement. Treating rat condylar chondrocytes⁸⁹ and bovine cartilage explants⁹⁰ with IL-1 α reduces levels of Prg4. Notably, this change in expression is rescued with TGF- β 1^{57,89} or BMP-7 treatment in monolayer culture⁵⁶. It has been demonstrated that IL-1 α reduces the F-actin network in isolated primary chondrocytes and increases expression of the mechanosensitive channel Piezo1 channel⁹¹. Interestingly, treatment with IL1- β changes the microstructure of the cytoskeleton including a loss in organization of tubulin and vimentin⁹². IL1- β changes chondrocyte cell morphology as cells condense their cell shape yet maintain F-actin protrusions⁹³. IL-1 was reported to increase F-actin in articular chondrocytes⁹⁴; this may be via Rho GTPase activity as both TNF- α and IL-1 activate Cdc42 in fibroblasts⁹⁵. Taken together, there is a connection between F-actin polymerization and inflammatory cytokines as shown *in vitro*.

1.6 Mechanical Stimuli as a Regulator of Prg4

Mechanical stimulation is critical for maintaining joint homeostasis. Prg4 expression requires different types of mechanical stimulation. In 2D and 3D bovine

chondrocyte cultures, Prg4 is upregulated under application of cyclic tensile strain⁹⁶. Movement of joints with natural loading stimulates Prg4; mice housed with running wheels were found to have higher levels of Prg4 expression in their knee joints than those without a running wheel, induced via a COX-2 dependent pathway⁹⁷. The same study also found shear stress increases in Prg4 to be through a CREB dependent manner via increased expression of parathyroid hormone related peptide (PTHrP) and prostaglandin 2 (PGE-2)⁹⁷. In larger scale joint models, continuous passive motion applied to bovine joints (allowing the articular surfaces to remain in contact and in motion) increases Prg4 synthesis and secretion⁹⁸. Bovine cartilage explants, under dynamic shear stimulation, secrete more Prg4 than those that undergo no mechanical stimulation or merely compression⁹⁹. Chondrogenic phenotype in fibrin-HA gels can be enhanced with the combination of multi-axial movement to mimic natural joint movement and fibroblast growth factor-18 (FGF-18) which leads to an increase in Prg4¹⁰⁰.

What connects mechanical stimulation to actin dynamics is not entirely known; how mechanical forces affect actin polymerization may be connected to ABPs and their affinity to F-actin filaments. Experimentally it has been shown to be regulated by protein kinase A (PKA) In water/solid interfaces mechanical forces can be used to induce both polymerization and depolymerization of F-actin. Polymerization is force-dependent; at low levels of force, G-actin polymerizes into F-actin while strong forces cause the F-actin to depolymerize¹⁰¹. Interestingly, the type of mechanical load also affects polymerization. Actin dissociates under dynamic loading, and to a lesser extent under static loading¹⁰². Regarding actin dynamics, differences in ABP affinity under different mechanical stimuli could affect regulation of Prg4. For example, the binding

rate of cofilin decreases when F-actin is tensed at a low level which ultimately results in a decrease in severing. The filament concentration itself affects affinity to cofilin; interconnected filaments are severed quicker than independent filaments¹⁰³.

1.7 Other Modulators of Prg4

Other modulators important in articular cartilage regulation have been found to modulate Prg4. HA has been shown to increase the proliferation of synovial cells from temporomandibular joint (TMJ) OA patients, as well as the expression of Prg4 in hypoxic conditions¹⁰⁴.

Prompted by a study by Decker et al. that found Kartogenin induces mesenchymal stem cells (MSCs) to differentiate into chondrocytes and increase levels of Prg4¹⁰⁵, Miyatake et al. investigated its effect on bovine knee joints and found it has no effect on the TGF- β 1 mediated increase of Prg4, nor that it prevents decreases in Prg4 production caused by IL-1 β ^{105,106}. This difference in noted effect on Prg4 may be due to the chosen cell type used, as MSCs may respond differently to Kartogenin compared to chondrocytes of bovine knee joints. Kartogenin has been shown to have no effect on cytoskeletal F-actin in fibroblasts¹⁰⁷.

Oxygen tension affects Prg4 where normoxic conditions are favored⁹⁶. Bovine superficial zone chondrocytes show decreased expression of Prg4 in hypoxic conditions and hypoxia suppresses TGF- β -mediated Prg4 expression^{96,108}. Interestingly, hypoxia reduces alpha smooth muscle actin in the presence of TGF- β 1¹⁰⁹.

Electromagnetic fields affect Prg4 metabolism. Rat chondrocytes exposed to a sinusoidal electromagnetic field (SEMF) express higher levels of Prg4, TGF- β 1 and

Smad2¹¹⁰. Knockout of transcription factor FOXO1 in mice leads to irregular articular cartilage and a decrease in Prg4 expression¹¹¹.

Oncostatin M (OSM) increases expression of Prg4 in bovine cartilage explants¹¹². Upregulation of OSM is found only within a subset of OA patients and is more related to rheumatoid arthritis however it may play a role in inflammatory OA¹¹³.

1.8 Actin Binding Proteins Regulate Chondrocyte Phenotype

Actin binding proteins (ABPs) regulate actin polymerization status and structure. In passaged chondrocytes, knockdown of cofilin, an actin depolymerizing factor, increases actin polymerization and collagen type I expression⁴¹. Adseverin, an actin capping and severing ABP, also regulates chondrocyte phenotype by maintaining the proportion of G- and F-actin as well as expression of the chondrogenic transcription factor Sox9, and aggrecan¹¹⁴. Proteomic analysis of chondrocytes derived from healthy patients vs those with OA reveals that F-actin depolymerizing proteins destrin and cofilin-1 are downregulated in OA, and depolymerizing agents cofilin-2 and gelsolin are upregulated. Differences in the actin dynamics of cofilin-1 and cofilin-2 suggest that changes in APB composition is critical in chondrocyte homeostasis compared to a diseased state.

Besides polymerization status, F-actin networks can respond to mechanical stimuli to regulate gene expression to maintain homeostasis¹¹⁵. SZCs require mechanical stimulation for normal homeostasis and a lack of shear stress reduces Prg4. It is suggested that normal homeostasis of SZCs requires shear stress to induce Prg4 and the actin cytoskeleton may play an essential role.

In tendon explants, stress deprivation leads to an increase in G-actin, a decrease in tenogenic markers, and an increase in matrix metalloproteinase-3. Interestingly, these changes were found to be recapitulated by inhibiting the actin stabilizing protein, Tropomyosin3.1 (Tpm3.1) which reduced F-actin and tenogenic expression¹¹⁶. The family of actin binding proteins Tropomyosins (Tpms) are considered master regulators of the F-actin cytoskeleton¹¹⁷. They are elongated proteins that directly bind along seven consecutive actin subunits on the long-pitch helix of actin filaments. After binding, Tpms polymerize end-to-end to stabilize F-actin as well as regulating affinity to various actin binding proteins that have different functions such as severing, capping, contractility, cross-linking, and depolymerizing¹¹⁸. There are over 40 isoforms of tropomyosin found in both muscle and non-muscle cells, and each can provide a different function in organizing complex structures and performing metabolic and locomotive activities; isoforms are not functionally redundant¹¹⁷. Regarding cartilage and the superficial zone, it remains vastly understudied how Tpms regulate chondrocyte phenotype in both isolated and native chondrocyte environments.

Various forms of tropomyosins can have opposing effects on actin dynamics insinuating that a delicate balance of tropomyosins is critical to maintain homeostasis. Furthermore, by targeting a specific Tpm with a known function, we can get specific actin formation. TPM1 genes regulate cofilin-dependent actin dynamics differently compared to TPM3 genes. Tpm1.6 and Tpm1.8 isoforms slow actin depolymerization. Tpm3.2 and Tpm3.4 isoforms increase actin depolymerization by increasing affinity to cofilin-1, a severing APB, which ejects tropomyosin isoforms from actin¹¹⁹. Jansen et al. found cells supplemented with Tpm isoforms 1.6, 1.7, 2.1, 3.1, and 4.2 had

increased F-actin filament length¹²⁰. Furthermore, they showed these isoforms decreased the severing rate of actin, and all but Tpm1.6 show an increase in the elongation rate of actin filaments¹²⁰. Opposing this, Janco et al. found that these Tpm isoforms decreased the rate of polymerization (except for Tpm2.1, which increased the rate by ~15%)¹²¹. In addition, Janco et al. found that Tpm1.1, Tpm1.8, Tpm1.12 decrease elongation rate; Tpm1.8 was the strongest inhibitor of the rate of elongation, with Tpm3.1 being the second¹²¹. This difference highlights the context specific of role Tpm play in actin polymerization in different biochemical assays.

Previously, the role of Tpm3.1 has been investigated in regulating cellular phenotype by promoting F-actin stress fibers in flattened lens epithelial cells as well as in elongated tenocytes^{116,122}. Tpm3.1 is critical in neurons for neuronal projections both in length and complexity¹²³. Tpm3.1 has also been studied as a target for anti-cancer treatments and has molecular inhibitors including ATM-3507 and TR100 which target the C-terminus of the protein^{124,125}. These drugs do not inhibit Tpm binding to F-actin rather prevent its polymerization along the barbed end. It is still being elucidated the exact mechanism of Tpm dynamics in cells in their native environment, and there remains contrary evidence for the specific functions of different isoforms.

1.9 Discussion

1.9.1 The Future of Cytoskeletal Research in Regulating Chondrocyte Phenotype

While there are limitations in studying actin structures in chondrocytes *in vivo*, previous studies have characterized actin organization in sections of tissue explants and *in vitro* isolated cell cultures. Actin structures of SZCs visualized in native tissue

en face have punctate actin around the cortex of cells^{36,126}. Like in native tissue, 3D cultured chondrocytes have actin cortically arranged⁵⁸. However, chondrocytes grown on monolayer tissue culture plastics assume stress fiber formation, an elongated cell shape which causes phenotypic changes⁴¹. For this reason, it is said that actin structure and function in chondrocytes is context specific based on the environment in which it is studied¹²⁷. Chondrocyte phenotype has been reviewed to be controlled by the balance between actin polymerization and depolymerization including the expression of Prg4³⁰.

As reviewed, much of what has been studied regarding regulation of Prg4 via the actin cytoskeleton and APB regulators utilizes *in vitro* monolayer culture and biochemical data solely. Furthermore, there are limited studies that connect modulation of Prg4 to F-actin arrangement despite evidence for strong regulation. It is necessary to study the actin cytoskeleton in SZCs ideally in physiologically and mechanically representative environments. The study to strive for is studying the actin cytoskeleton *in vivo*, although this poses technical challenges in methodology development. Hence, we identify the need to develop novel imaging methodologies to study actin in native cell environments. Existing tools include GFP-actin expression which has been validated to have minimal effect on chondrocyte phenotype and can therefore be used to characterize live actin dynamics⁵⁸. GFP-actin chondrocytes have been used in 3D culture to examine the real time effects IL-1 α and TGF- β have on cytoskeletal organization⁵⁸.

In chondrocyte phenotype, what remains uncharacterized is the organization of actin in the native SZCs. Future studies should aim to delineate if actin is dynamic in native SZCs and if SZC actin dynamics play the same role *in vivo* as it is *in vitro*.

1.9.2 Unanswered Questions on the Regulation of Prg4 via Actin

Current management strategies and surgery are not sufficient to prevent OA completely and no cell-based therapies exist. In the case of post traumatic Osteoarthritis (PTOA) after injury, there is a need to identify pharmacological targets for the development of novel OA therapies. If future studies can delineate the regulation of Prg4 via the actin cytoskeleton in native tissue, this may shift the treatment options for patients who are at risk of developing OA after an injury.

It has previously been thought that since SZCs are flatter and less round than DZCs, 2D culture environments that promote flattening are more conducive to Prg4 production and SZC phenotype, however this is being contradicted by a body of evidence^{96,128}. Furthermore, because actin regulation of phenotype is culture context specific¹²⁷, it remains unknown if actin dependent regulation of Prg4 occurs in SZCs in their native matrix environment. Furthermore, it also remains uncharacterized how other actin binding proteins regulate this process, such as Tropomyosins (Tpms). If the specific actin binding protein regulates a certain formation of actin in chondrocytes that is favorable for proper gene expression, this could potentially be a therapeutic target for OA prevention.

Chapter 2

***EX VIVO* CULTURE OF NATIVE MOUSE FEMORAL HEAD CARTILAGE LEADS TO AN IMBALANCE IN MATRIX HOMEOSTASIS MRNA LEVELS**

2.1 Abstract

Osteoarthritis (OA) is an irreversible, debilitating disease with poor long-term reparative outcomes. OA pathogenesis involves the dysregulation of matrix homeostasis favoring cartilage catabolism which leads to the degradation of articular cartilage. In OA, functional annotation of differentially expressed genes shows the greatest changes in the actin cytoskeleton pathway. Previous studies have shown that the actin cytoskeleton regulates chondrocyte phenotype, however, these studies are based on isolated cells *in vitro*. Since actin regulation of phenotype is context-specific, we aimed to study the regulation of actin and chondrocyte phenotype in the native tissue environment. In this study, we developed a whole organ culturing methodology that may be sufficient to study regulation of OA-like gene expression changes via actin polymerization status. We found that culturing native cartilage of the mouse femoral head with no added growth factors results in gene expression changes that recapitulates aspects of an OA phenotype including a decrease in cartilage matrix and an increase in degradative enzyme mRNA levels. We also characterize dramatic increases in inflammatory marker mRNA levels including tumor necrosis factor alpha (TNF- α). Furthermore, we show evidence of actin reorganization over time in culture, which could be regulated by changes in a select number of actin binding proteins such

as gelsolin (Gsn) and cofilin (Cfl). This model could give insight into the regulatory drive of OA-like changes via the actin cytoskeleton.

2.2 Introduction

Osteoarthritis (OA) involves the dysregulation of articular cartilage homeostasis leading to cartilage degradation. OA disease progression is not fully understood; interplay between inflammation and the maladaptive repair system of articular cartilage make elucidation difficult¹²⁹. In patients, OA most commonly affects weight-bearing joints such as the hip and knee with varying presentation^{130,131}. While pain caused by OA in these joints can be relieved by total joint replacements, no therapeutic interventions exist to protect patients from developing OA.

In healthy articular cartilage, the sole resident cells, chondrocytes, function to maintain extracellular matrix (ECM) turnover by producing components such as collagen and proteoglycans¹³². The building block of healthy articular cartilage ECM is collagen type II (COL2) which provides tensile strength. Collagen types I, IV, V, VI, IX, and XI are also present in a lower proportion that stabilize the COL2 network¹. To hydrate the tissue for protection against compressive forces, healthy articular cartilage has a large amount of aggrecan (Acan) in its matrix¹. Progressive degradation of Acan is a hallmark of OA¹³³.

Before large-scale degradation of cartilage begins, there is a dysregulation of gene expression which can warrant biomarkers and modulators of OA. An increase of proinflammatory cytokines in the blood of patients with OA include interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), and interleukin-6 (IL-6)¹³⁴ which down regulate ECM synthesis and increase ECM degradative enzymes in articular cartilage^{135,136}. TNF- α induces aggrecanase and matrix metalloproteinase (MMP)1, 3

and 13 expression^{136,137}; inhibition of TNF- α receptors has a protective effect against OA through this mechanism¹³⁸. IL1- β also increases Mmp-1, Mmp-3 and Mmp-13 in chondrocytes¹³⁹ and decreases Timp1 expression. Il1- β also plays a role in maintaining cartilage as deletion of the Il1- β gene accelerates cartilage degradation¹⁴⁰. IL-6 is found in higher concentrations in the synovium of patients with OA¹⁴¹. and the IL-6 receptor is increased with aging¹⁴². Changes in catabolism and inflammation go hand in hand; within 6 hours of surgical induction of OA in mice, upregulated genes include Il-1 β , Il-6, Mmp3, Adamts5, and Adamts1¹⁴³.

Pivotal degradative enzymes that are responsible for matrix degradation in OA include MMPs, a disintegrin and metalloproteinase with thrombospondin motifs (Adamts) family of proteinases, and cathepsins (Cts) from the papain superfamily of cysteine proteases. MMP13 is the major MMP responsible for cleaving COL2 in cartilage^{144,145}. Mmp3 is significantly increased in OA pathogenesis¹⁴⁶ and is stimulated by both overloading and underloading¹⁴⁷. Tissue inhibitors of MMPs (TIMPs) inhibit activity of MMPs; maintaining a balanced TIMP to MMP ratio attenuates articular cartilage damage^{139,148}. Adamts1 expression is significantly upregulated in OA cartilage¹⁴⁹ while Adamts5 is the major aggrecanase in OA¹⁵⁰ as best illustrated by Adamts5 KO mice which have a chondroprotective effect¹⁵¹. The same study also identified that altered expression of Col1 requires Adamts5 activity. Another family of proteases, cathepsins, are involved in collagen and aggrecan degradation, bone reabsorption, in articular cartilage notably cathepsin B (CTSB), cathepsin D (CTSD), and cathepsin K (CTSK). CtsB activity is enhanced in areas of active cartilage disease and declines in expression in advanced OA^{152,153}. CTSD

directly cleaves aggrecan in articular cartilage¹⁵⁴. CTSK inhibitors have been identified for development of OA therapies¹⁵⁵⁻¹⁵⁷.

Although cartilage lacks the ability to sufficiently self-repair, molecular modulators secreted by chondrocytes provide a chondroprotective effect against cartilage damage. Clusterin (Clu), a cytoprotective molecule attenuated by proinflammatory cytokines¹⁵⁸, is expressed in high levels in early OA however, it is reduced in advanced OA cartilage¹⁵⁹. The surface lubricant proteoglycan 4 (Prg4) is a chondroprotective molecule found to be downregulated in early OA models^{20,21,27,160}. It is also downregulated in response to inflammatory mediators IL1- β and TNF- α ¹¹².

The arrangement of F-actin has been shown to regulate chondrocyte phenotype, although its exact implication in OA remains unknown^{41,161}. In OA, transcriptional analysis of differentially expressed genes shows the most affected pathway in chondrocytes is the actin cytoskeleton^{42,43}. F-actin itself is less prominent in OA chondrocytes⁹¹. While the connection between actin and gene regulation has been elucidated, the regulatory mechanisms of F-actin depolymerization in chondrocytes is not completely known. Actin binding proteins (ABPs) with severing/depolymerizing functions promote disassembly and F-actin network reorganization. In cultured chondrocytes, it has been shown that gelsolin (GSN) and adseverin (ADS), actin capping and severing proteins, regulate actin polymerization status *in vitro*¹¹⁴. ADS maintains the proportion of G-/F-actin which is key for Sox9 and Acan expression¹¹⁴. Loss of Ads expression enhances OA severity by enhancing chondrocyte hypertrophic differentiation (Chan, Science Advances, in review). Furthermore, in passaged chondrocytes, knockdown of the actin depolymerizing factor cofilin (Cfl) decreases actin polymerization and increases Col1 expression⁴¹. In human

OA chondrocytes, Cfl1 is downregulated and Gsn and Cfl2 are upregulated suggesting actin polymerization status in OA may be mediated by these ABPs. A key in human OA is also the stiffening of the matrix¹⁶² which affects chondrocyte phenotype. This is exemplified by dedifferentiated chondrocytes due to substrate stiffness with an increase in actin polymerization, and a contractile phenotype. This has been found to be regulated by myocardin-related transcription factor-a (MRTF-A), in passaged chondrocytes. Inhibition of MRTF-A, which has a high affinity for G-actin, found reduced expression for α -smooth muscle actin (α -sma) and transgelin (Tagln) which coincided with inhibition of gel contraction¹⁶³. There is a lack of models sufficient to study gene regulation of OA via the actin cytoskeleton, although existing models have been used each with their own costs and benefits.

To gain a better understanding of OA pathogenesis, both *in vivo* and *ex vivo* models have been used each with their own advantages and disadvantages¹⁶⁴. For *in vivo* models, both large and smaller animal models have been employed. While large animal models may better mimic the human condition than rodent models, they are costly. Rodent models, particularly mice, are commonly used due to cost and ease of maintenance as compared to larger animals. Importantly, mice are also genetically tractable. OA is typically induced by surgical or chemical means. However, spontaneous (aging or genetic) models have also been employed. OA is usually studied in the knee joint which in mice is only a few cell layers thick. Other OA models that study OA in the hip also exist¹⁶⁵, which affords a greater volume of tissue sample. The study of OA using *in vitro* cell cultures models provides more throughput examination, which is particularly helpful when studying gene regulation. A large quantity of cells can be isolated from cartilage of joints from larger species, including

human. OA processes can be simulated using cytokines, often IL-1, TNF- α , or IL-6. Additionally, pharmacological, or genetic approaches can be used to manipulate isolated cells to study gene regulation of OA. A major disadvantage is that cells in monolayer culture are removed from the *in vivo* environment and lose influence of the native extracellular matrix. Cells in monolayer culture rapidly undergo dedifferentiation⁴¹ which is particularly limiting when studying how the actin cytoskeleton regulates OA, which immediately reorganizes when chondrocytes are isolated.

Cartilage explant culture is an excellent alternative to *in vitro* cell culture studies. In explant studies, cartilage tissue is harvested from joints, typically of larger species. While human cartilage explants femoral heads have been used for *ex vivo* studies^{166,167}, availability of healthy human tissue is a limitation. OA induction is achieved in explants via cytokine stimulation, injury, or mechanical loading to the joint¹⁶⁸. A major advantage over *in vitro* cell culture models is that chondrocytes are maintained in their natural extracellular matrix.

In this study, we develop a cartilage explant culture of the mouse femoral head that gives the benefit of studying specific gene regulation in the native chondrocyte environment. We develop a whole organ culture using this joint to induce certain aspects of gene expression modulation in OA. and to correlate these changes with F-actin organization and polymerization status. We determine that culturing femoral heads in the absence of exogenous cytokines or growth factors models certain mRNA changes that occur in OA.

2.3 Materials and Method

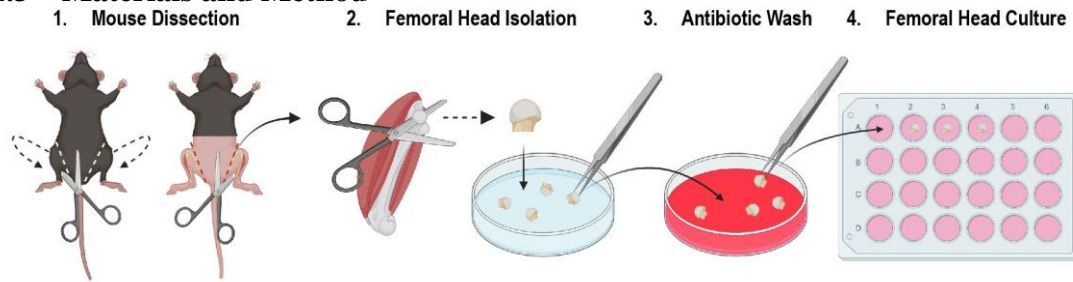


Figure 2.1. Mouse femoral head dissection methodology beginning with mouse dissection, femoral head isolation, antibiotic wash, and femoral head culture.

2.3.1 Mice and Tissue Isolation

Wild-type mice (C57BL/6J background) obtained from Jackson laboratories, were used for femoral head isolations, between the ages of 8-10 weeks old. Experiments were conducted following approved animal protocols by the University of Delaware and IACUC.

2.3.2 Femoral Head Dissection, Isolation, and Culture

Femoral heads were removed from the mice as described previously¹⁶⁹. Euthanized mice are laid on their backs and their bottom half is sprayed liberally with 70% (vol/vol) ethanol. Small dissecting scissors were used to cut the skin around the abdomen; skin was pulled off gently to expose the muscle of the lower torso (Figure 2.1). Muscle is cut away from the femoral head joint and held with dissecting scissors to dislocate the hip joint. The remaining muscle is cut away and the mouse leg is separated from the body at the hip, exposing the femoral head. To isolate native femoral head tissue, a cut is made directly below the femoral head where bone remains intact with cartilage of no more than 1 mm of the femur. Extra connective tissue and

fat is removed as best as possible; the ligamentum teres remains intact. Immediately after isolation, femoral heads were placed in a petri dish containing 1x phosphate-buffered saline (PBS; 190 GenClone). Samples for freshly isolated are then dissected and cultured samples are prepared for tissue culture.

In a sterile biosafety cabinet, femoral heads were washed in a petri dish filled with 25 mL of DMEM with 1% Antibiotic Antimycotic (AMAB) and incubated at 37°C. After 15 minutes, individual femoral heads were placed into wells of a 24 well plate; each well contained 1mL of serum-free DMEM with 1% AMAB at 37°C.

2.3.3 RNA Extraction and PCR

To isolate cartilage from femoral head explants for mRNA analysis, femoral heads were transferred and placed in a petri dish consisting of PBS. Under a dissection microscope, a scalpel with a blade size 15 is used to cut off the ligamentum teres from the bone along with forceps to hold the femoral head in place. Cartilage is dissected from the femoral head by cutting off slices of cartilage from bone.

Cartilage from 2-4 femoral heads were pooled together for each sample; cartilage is stored in 1.5mL Eppendorf tubes with 1 mL of PBS. After collection of all femoral head cartilage, PBS is swapped for either 500 μ L (2 femoral heads) or 1000 μ L (4 femoral heads) TRIzol (Sigma-Aldrich, Burlington, MA). Cartilage was crushed manually (~5 minutes for each sample) using a Pellet Pestle until there were no large visible pieces. Samples of crushed cartilage in TRIzol were stored at -20 °C until RNA extraction was proceeded. To separate RNA, chloroform was used for phase separation followed by selective recovery of total RNA using an RNA clean-up kit (RNA Clean & Concentrator-5; Zymo, Irvine, CA). RNA was then reverse transcribed to cDNA using the UltraScript 2.0 cDNA Synthesis Kit (PCR Biosystems, Wayne,

PA). Real time RT-PCR reactions were run on a Cielo 3 PCR machine (Azure; Houston, TX, USA). Gene expression was normalized to relative Ct values of housekeeping genes 18S or GAPDH values. For each set of experiments, the control group (freshly isolated) was normalized to 100% and the experimental group (2-day culture) was expressed as a percentage of the control. All PCR experiments were performed on at least 3 separate occasions (n=8) with femoral head cartilage from different mice.

2.3.4 Femoral Head Sagittal Sectioning, Immunohistochemistry, and Confocal Imaging

Femoral head tissue was fixed overnight in 1 mL of 4% paraformaldehyde (PFA) for F and G-actin staining. After a 3x2 PBS wash (touching fresh PBS briefly twice, waiting 5 minutes, repeated 3 times), tissue was placed in 30% sucrose solution overnight. Another 3x2 PBS wash was employed and then tissue was embedded in OCT from Tissue-Tek®. Embedded tissue was stored at -80°C until sectioned using the Leica Cryostat CM3050. 12 µm sagittal sections were obtained with the inside temperature of the cryostat at -20°C with the cartilage side cut by the blade first. Serial sections were collected on Fisherbrand charged 25 × 75 mm slides. Slides were stored at -20°C until prepared for staining.

For immunohistochemistry, slide sections were placed in a slide staining tray from Heathrow Scientific (HEA15951A) with the deep well filled with 1x PBS. Sections were permeabilized prior to staining with 1 drop of permeabilization/blocking buffer (PBS containing 0.3% Triton, 0.3% bovine serum albumin, and 3% goat serum) for 30 minutes at room temperature. For actin visualization, F-actin was stained with 1:50 rhodamine-phalloidin (Biotium); G-actin,

1:400 Deoxyribonuclease I (DNase-I) conjugated with Alexa Fluor™ 488 (Invitrogen, Waltham, MA); and nuclei, 1:500 Hoechst 33342 (Biotium) in a permeabilization/blocking buffer solution for 1 hour at room temperature. Stains were washed with 3x2 PBS wash prior to adding 1 drop of ProLong®Gold Antifade Reagent (#9071, Cell Signaling Technology, Danvers, MA) and sealing with coverslips.

Stained sections and whole tissues were imaged using a Zeiss LSM880, AxioObserver laser-scanning confocal fluorescence microscope (Zeiss) equipped with a 20x 0.8 NA objective; Z-stack images were captured with a step size of step size 0.5 μm . The pinhole size was 1 Airy Unit (AU), and maximum intensity projection images of Z-stack images were processed.

2.4 Results

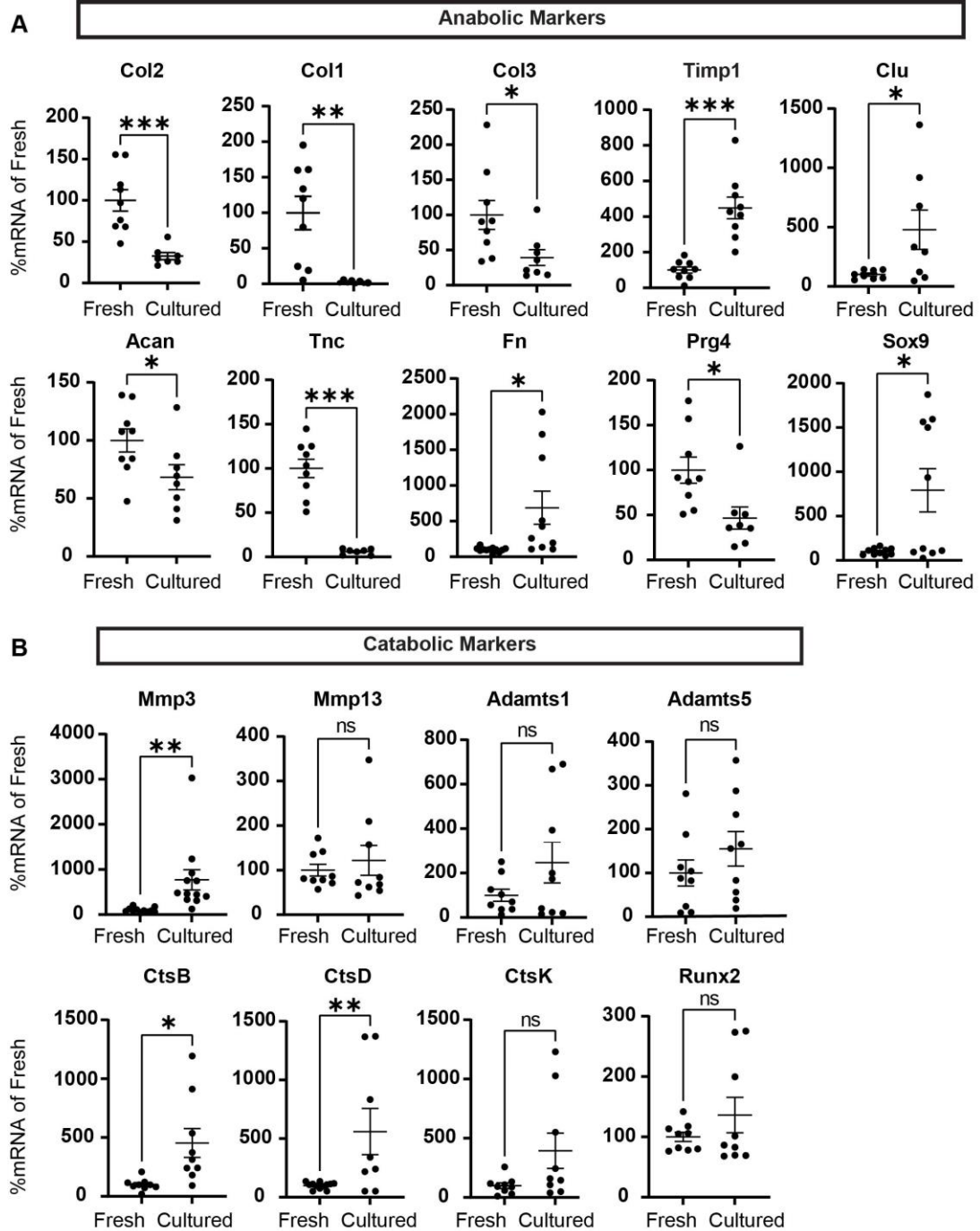


Figure 2.2 Culturing leads to changes in matrix mRNA levels favoring catabolism. [A] Anabolic markers decrease in culture. [B] The degradative enzymes Mmp3, CtsB, and CtsB mRNA levels increase in culture.

2.4.1 Culturing mouse femoral head cartilage in serum starved DMEM leads to tip in matrix homeostasis favoring catabolism

Isolation of mouse femoral heads from its native joint environment and culturing in serum starved DMEM conditions results in changes in ECM mRNA levels that resemble an OA-like phenotype. We see a broad decrease in select collagen mRNA levels (Col1, Col2, Col3) and the healing marker Tnc (Figure 2.2A). However, another fibrocartilage marker of healing degenerated cartilage is up; fibronectin (Fn) mRNA levels are found significantly increased (Figure 2.2A). Further evidence of an induced protective response is found in our model; mRNA levels for Timp1 and Clu significantly increase (Figure 2.2A). mRNA levels of proteoglycans Acan and Prg4 decrease (Figure 2.2A). Interestingly, we see an increase in mRNA levels of SRY-Box Transcription Factor 9 (Sox9) which is a known transcription factor of chondrogenic genes like Col2 and Acan¹⁷⁰ (Figure 2.2A).

Significant increases in catabolic markers mRNA levels include Mmp3, CtsB, and CtsD (Figure 2.2B). No significant change was observed in Mmp13 or CtsK mRNA levels. Nor did we see increases in mRNA levels of either Adamts1 or Adamts5 the latter being the major aggrecanase in mouse cartilage¹⁷¹. Runt-related transcription factor 2 (Runx2), a marker for developing bone¹⁷², does not change in mRNA levels (Figure 2.2B). Taken together, our comparison of mRNA levels from two days in culture shows a tip in matrix homeostasis favoring catabolism and a possible attempt to repair.

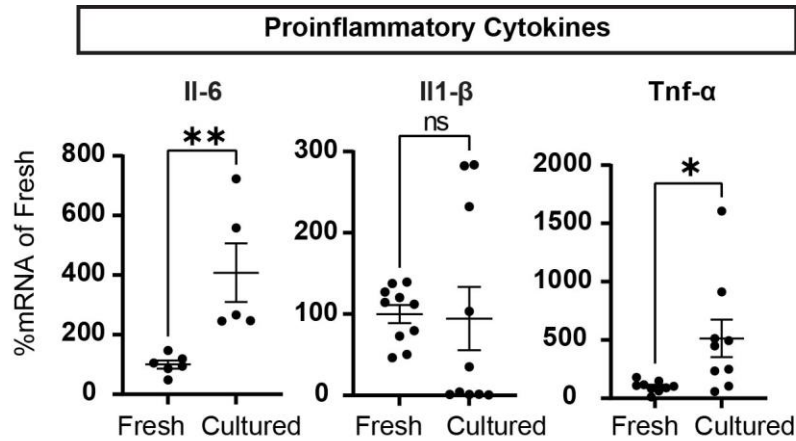


Figure 2.3 Proinflammatory cytokine mRNA levels increase in culture conditions (IL-6 and Tnf- α).

2.4.2 Culture conditions increase mRNA levels of IL1- β and TNF- α

We wanted to probe inflammatory cytokines produced by the articular cartilage during culture which may play a role in mediating the change in catabolic and anabolic factors. Under inflammatory conditions, chondrocytes produce more catabolic factors and attenuate their expression of anabolic matrix molecules. In our model, IL-6 and Tnf- α mRNA levels are found to be elevated in femoral heads that are exposed to culture conditions for 2 days (Figure 2.3). We reported no changes in IL1- β mRNA levels.

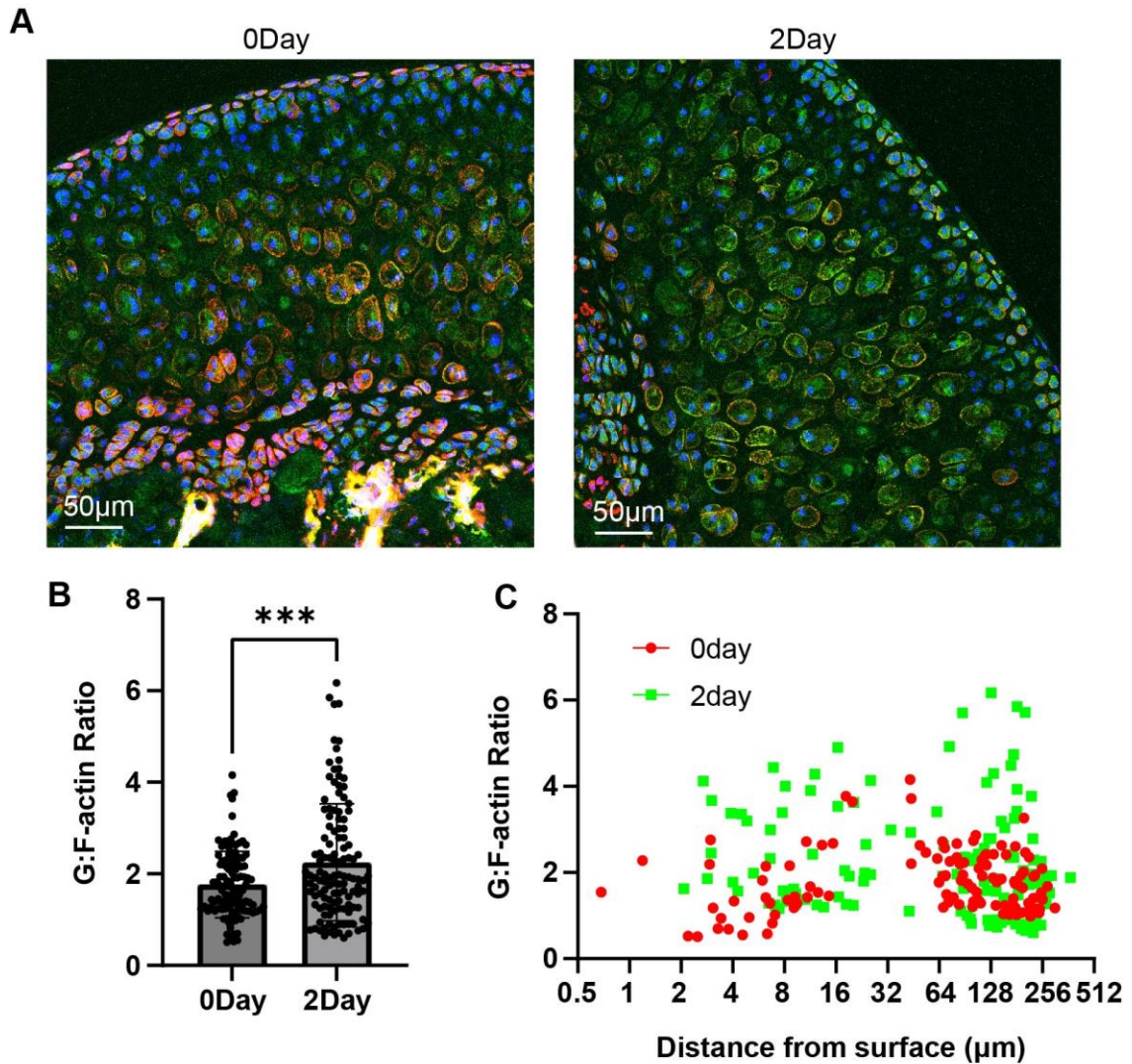


Figure 2.4 Actin polymerization decreases in culture. [A] Sagittal sections of femoral head culture stained with Phalloidin for F-actin (red), DNase-I for G-actin (green), and Hoechst for nuclei (blue) show reduced F-actin staining after 2 days in culture. [B] The G:F-actin ratio of chondrocytes increases after 2 days in culture. [C] G:F-actin ratios of individual cells circled from sections plotted against distance from surface shows the decrease in F-actin occurs throughout the tissue as well as in cells close to the surface.

2.4.3 The F-actin cytoskeleton is altered in culture

In OA, there are changes in the cytoskeleton compared to healthy chondrocytes that include tubulin and vinculin differences as well as differences in cytoskeleton binding proteins⁹². Furthermore, under inflammatory conditions, F-actin becomes reduced in the presence of inflammatory cytokines and is rarified in human OA patients^{58,91}. To examine F-actin reorganization in our *ex vivo* whole organ culture system, we visualized F- and G-actin in sagittal sections of native femoral head cartilage (n=1). We determined that culture of the mouse femoral head leads to F-actin reorganization in native chondrocytes. We see an increase in fluorescent staining for G-actin and a decrease in fluorescent staining of F-actin (Figure 2.4A). This is reflected in the G:F-actin ratio which is significantly increased in the cultured femoral head (Figure 2.4B). To determine where this change occurs, we plotted G:F-actin as a function of distance from the surface (Figure 2.4C). We see a change in G:F-actin throughout the tissue as well as apparent in cells close to the surface. What regulates this change is undetermined by this analysis alone, however, changes in actin binding protein mRNA levels may give insight into regulation of the F-actin cytoskeleton in relation to an OA phenotype. No significant changes were observed in mRNA levels of *Ads*, *Tagln*, and α -*sma*, however we see an increase in *Gsn* mRNA levels and a decrease in *Cfl* mRNA levels (Figure 2.5).

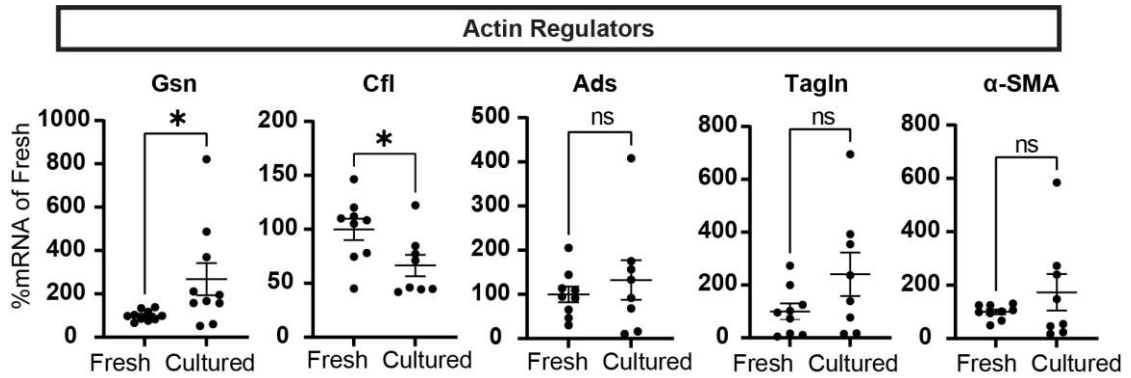


Figure 2.5 Actin binding protein mRNA levels of Gsn and Cfl are altered with culture.

2.5 Discussion

We characterize native cartilage in *ex vivo* culture that models certain, but not all aspects, of mRNA level changes seen in OA changes. To validate these changes are not occurring due to cell death in tissue culture, we performed live dead assays to confirm chondrocyte viability (data not shown). We characterize this tissue culture to show an imbalance of some changes in matrix homeostasis mRNA levels and a change in inflammatory mRNA levels. These changes correlate with a change in G:F-actin over time in culture. We demonstrate mRNA level changes that model some aspects of an OA-like phenotype in a system that allows for the study of gene regulation via F-actin.

A decrease in collagen mRNA levels is the opposite of what is usually found in metabolically active chondrocytes; increased collagen production has been documented in OA^{173,174}. Although, it is generally accepted that the expression of Col2 decreases over time in OA¹⁷⁵. In fact, it has been postulated that an upregulation of matrix genes in OA is a sign of a repair matrix^{42,176,177}. While Col2 is the main collagen that makes up the articular cartilage matrix, Col1 is a component of

fibrocartilage which is upregulated in OA. While an OA phenotype includes increased Col1 and Col3 expression in the creation of a repair matrix, why we may see a decrease in collagens is because what drives collagen production in articular cartilage is growth factors which are absent in our culture system^{178,179}. Col3 and Fn are also found to be highly expressed in areas of cartilage degeneration^{180,181} as part of a repair matrix. Fragmented Fn itself stimulates collagen and proteoglycan synthesis¹⁸². Tenascin-C (Tnc) is another matrix molecule that is upregulated after joint injury; Tnc is normally present in low levels in healthy articular cartilage and upregulated and stimulated by proinflammatory cytokines^{183,184}. Surprisingly, while Acan and Col1, 2, and 3 mRNA decreased, which are direct targets of Sox9, we did not see a change in Sox9 mRNA levels. While decreased mRNA levels is not an indicator of decreased transcriptional activity, this may be an indication of regulation of Col2 and Acan via other pathways than Sox9 transcription.

Cartilage degradation is mediated by degradative enzymes, which is a hallmark of OA¹⁸⁵. Mmp3 is the most strongly expressed Mmp in human osteoarthritic cartilage which decreases over time in OA^{186,187}; we see a significant increase in Mmp3 mRNA levels which is aligned with an OA phenotype in mice and human¹⁸⁸. We reported Mmp13 mRNA levels to remain unchanged, as well as Adamts1 and 5 which stray from an OA phenotype^{185,187}, showing not all aspects OA are induced. Alongside this, CtsB and CtsD mRNA levels increase with culture as well but not CtsK^{187,189}. The increase in matrix degradative enzymes may be in response to inflammation as a driver of OA^{144,186,190}.

Increases in inflammation, such as we find with an increase in Il-6 and Tnf- α , mRNA levels are consistent with other OA models. Furthermore, a decrease in F-actin

in our culture system is congruent with what has been characterized about F-actin under OA conditions^{58,91}. Surprisingly, the increase in Gsn and decrease in Cfl correlate with a decrease in G:F-actin in chondrocytes in a cultured femoral head for 2 days. These molecules may be the regulators of the reorganization of F-actin we see happening in 2 days in culture; knockdown of Cfl in isolated cells leads to increased polymerization so we could be observing the opposite effect in native chondrocytes⁴¹.

The limitations of this study include the undetermined driving forces of the modulated mRNA levels and G:F-actin. We postulate the driving forces for inducing these OA-like changes and actin reorganization are a lack of growth factors, lack of mechanical stimulation, changes in oxygen, osmotic stress, and trauma to the cartilage from the isolation may be jumpstarting a change in chondrocyte metabolic state. While it remains unclear what zone of articular cartilage these changes are occurring, the change in G:F-actin ratio appears to be throughout. Our model may show OA in the superficial zone of articular cartilage as we see decreases in SZ specific genes, like Col1 and Prg4. This could mean that we are modeling mRNA level changes in OA beginning in the SZ which is consistent with how this presents in patients^{4,5}. Upregulation of Tnc and Fn suggests an early stage of OA of our model as these molecules show focally enhanced deposition in upper fibrillated articular cartilage¹⁹¹ and Tnc has been shown to increase throughout all zones in severe OA¹⁹².

There is a need for *ex vivo* native tissue models of OA because it allows us to understand regulation of phenotype, elucidate molecular targets, and test drug abilities for OA prevention. A benefit to our model is that we do not rely on the addition of inflammatory cytokines to insight an OA like response so this may be able to be used in parsing out the effect of cartilage dysregulation without cytokine exposure.

Isolation of native mouse joints for an OA model allows for a greater sampling and broader exploration of mechanisms that regulate OA to allow for the development of novel therapies. The efficacy of molecular modulators can be used in culture to see attenuation of changing mRNA levels from the effect of culturing.

Chapter 3

F-ACTIN REORGANIZATION IS A REGULATOR OF PRG4 IN NATIVE MOUSE FEMORAL HEAD SUPERFICIAL ZONE CHONDROCYTES

3.1 Abstract

Osteoarthritis (OA) is an irreversible, debilitating disease that involves the dysregulation of matrix homeostasis. The initial stage of OA includes a decrease in proteoglycan-4 (Prg4) secreted by superficial zone chondrocytes (SZCs) to maintain cartilage lubrication. Prg4 itself is chondroprotective; supplementing joints with Prg4 prevents OA onset. Functional annotation of differentially expressed genes reveals the primary pathway affected in OA is the chondrocyte filamentous (F-) actin network. While *in vitro* culture studies have shown a strong regulation of Prg4 by actin, it remains unclear if F-actin reorganizes and regulates Prg4 in native cartilage. What regulates F-actin networks in native SZCs also remains unknown. F-actin networks are found in other cell types to be regulated by Tropomyosins (Tpms) which are rod-like proteins that regulate actin networks by binding alongside F-actin filaments to stabilize them. Tpm3.1 is found to stabilize stress fibers in lens epithelial cells and native tendon. We test the hypothesis that the reorganization of F-actin in native SZCs regulates Prg4 expression.

Key Words: Native Cartilage, Superficial Zone Chondrocytes (SZCs), Filamentous (F-) actin, Proteoglycan-4 (Prg4), Tropomyosins

3.2 Introduction

Prg4 is critical in maintaining joint homeostasis as it lubricates the articular surface and has a cytoprotective role. Secreted to the articular surface of cartilage, Prg4 makes up a lubricating component of synovial fluid playing a role in maintaining joint homeostasis by lowering the coefficient of friction between joint surfaces as well as antagonizing abnormal cell growth in the synovium¹. One of the first changes in OA progression is cellular changes in the superficial zone. Superficial zone loss of Prg4 expression and secretion may be an early contributor to OA⁴. Prg4 is found to be downregulated early in OA in both sheep and rodent models^{20,21,160}. Prg4 has a chondroprotective effect on cartilage; supplementing joints with Prg4 has been shown to prevent the onset of OA^{27,193}. Therefore, understanding the molecular underpinnings that regulate Prg4 may lead novel therapeutic interventions to prevent OA progression.

The actin cytoskeleton is a critical regulator of chondrocyte phenotype including the regulation of the SZC phenotype. Actin is an abundant cytoskeletal protein that makes up microfilaments¹⁹⁴. It exists as a monomeric unit, globular (G-)actin, that polymerizes to form filamentous (F-)actin. F-actin organizes into higher order structures that determine cellular shape and size. In native healthy chondrocytes, F-actin is found cortically arranged with reports of diffuse and evenly distributed around the periphery of cells^{36,37,195-197}. In OA, the actin cytoskeleton has been documented to have less prominent F-actin arrangement and has been found to decrease in the presence of inflammatory cytokines^{58,91}. However, it remains unclear how F-actin reorganizes in native chondrocytes. In SZCs specifically, there is a large pool of soluble G-actin in proportion to F-actin, and greater proportion F/G-actin in SZCs suggesting a cellular zone rich for actin remodeling^{36,38}. The strong regulation of SZC phenotype of Prg4 expression is based on 2D cultures of isolated SZCs^{31,32,38}.

Because actin is culture context specific, meaning F-actin regulates cell phenotype based on the extracellular environment¹²⁷, it remains unclear if actin dependent regulation of Prg4 occurs in SZCs in their native matrix environment. Additionally, it also remains uncharacterized how other actin binding proteins regulate this process, such as Tropomyosins (Tpms).

The family of actin binding proteins, Tpms, are considered master regulators of the F-actin cytoskeleton¹¹⁷. Tpms bind alongside F-actin filaments to stabilize them and control affinity of other actin binding proteins to actin filaments¹⁹⁸. There are over 40 isoforms of Tpms, and each provide a different function in organizing complex structures and performing metabolic and locomotive activities; isoforms are not functionally redundant by associating with different populations of actin filaments¹⁹⁹. Within native mouse cartilage, it remains uncharacterized which Tpm isoforms are present and regulate chondrocyte phenotype. In native tendon, Tpm3.1 associates with stress fibers, and regulates tendinosis-like gene expression in isolated tenocytes¹¹⁶. In lens epithelial cells, Tpm3.1 is critical in transforming growth factor beta (TGF- β) epithelial to mesenchymal transition in immortalized mouse lens epithelial cells¹²². We have confirmed expression of Tpm3.1 in native mouse cartilage using semiquantitative PCR. Tpm3.1 has molecular inhibitors including ATM-350727 which can be used to probe its regulatory role¹²⁴. In this study, we identify a need for high resolution imaging of F-actin networks in native SZCs to test the hypothesis that Prg4 is regulated via F-actin reorganization. Furthermore, we investigate the novel roles of Tpm3.1 as a regulator of Prg4.

3.3 Materials and Methods

3.3.1 Mice, Tissue Isolation, and Whole Femoral Head Explant Culture

Wild-type mice and Tpm3.1 knockout mice (Tpm3/ Δ exon9d^{-/-}), 8-10 weeks old, in the C57BL/6J background from JAX laboratories were used in this study. All experiments were conducted following approved animal protocols by the University of Delaware and IACUC. Following euthanasia, femoral head cartilage from both hips were removed from the joint cavity with the bone intact as previously described (10.1038/nprot.2010.179, Stanton). Whole explant culture of femoral heads was performed aseptically (Chapter 2). Immediately following isolation, femoral heads were immersed in 1x phosphate-buffered saline (PBS; 190 GenClone) and transferred into a sterile tissue culture biosafety cabinet.

3.3.2 Tissue Culture of Mouse Femoral Heads and Drug Treatment

Under sterile conditions, femoral heads were washed with DMEM with 1% Antibiotic Antimycotic (Millipore Sigma; Burlington, MA) and incubated at 37°C. After 15 minutes, femoral heads were placed in 1 mL serum-free DMEM containing 1% antibiotic/antimycotic (Corning; Manassas, VA) within wells of a 24 well plate with one femoral head per well. Femoral heads were cultured for up to 2 days. For TGF- β -2 treatment, 20 ng/ μ L of recombinant mouse TGF- β -2 (R&D Systems #7346-B2; Minneapolis, MN) was used as we determined that this concentration significantly increased Prg4 mRNA levels.

3.3.3 Fixation of Native Cartilage and Immunostaining of Sagittal Sections

For staining of F-/G-actin and Tpm3.1 immunostaining, femoral heads were fixed in 4% paraformaldehyde (PFA). For PRG4 staining, femoral heads were fixed

overnight in 1 mL of 100% methanol. Following fixation, tissues were washed in PBS and then placed in 30% sucrose solution at 20°C. After an overnight incubation, tissues were embedded in OCT (Tissue-Tek®, city) and immediately frozen on dry ice. Embedded tissue was stored at -80°C until sectioning. Sectioning was performed using the Leica Cryostat CM3050). 12-micron sagittal sections were obtained with the internal cryostat temperature at -20°C. Serial sections were collected on charged 25 × 75 mm slides (Fisherbrand 12-550-143; Waltham, MA).

For immunohistochemistry, slides were placed in a humidified slide staining tray (Heathrow Scientific #HEA15951A; Vernon Hills, IL). Sections were permeabilized with 1 drop of permeabilization/blocking buffer (PBS containing 0.3% Triton, 0.3% bovine serum albumin, and 3% goat serum) for 30 minutes at room temperature. Primary antibody staining was performed overnight at 4°C. After incubation in primary antibody solution, sections were washed six-times in PBS. Secondary antibody solutions containing fluorescent secondary antibodies and nuclear counterstain (Hoechst 33342) were placed on sections, and then sections were stored in dark conditions. F-actin visualization was achieved by staining with 1:50 rhodamine-phalloidin (Biotium, San Francisco, CA); G-actin, 1:400 Deoxyribonuclease I conjugated with Alexa Fluor™ 488 (Invitrogen, Waltham, MA); and nuclei, 1:500 Hoechst 33342 (Biotium) in a permeabilization/blocking buffer solution. After 1h incubation at room temperature, slides were washed six times in PBS and coverslip mounted using ProLong®Gold Antifade Reagent (#9071, Cell Signaling Technology, Danvers, MA).

Stained sections and whole tissues were imaged using a Zeiss LSM880, AxioObserver laser-scanning confocal fluorescence microscope (Zeiss) equipped with

a 5x 0.25 M27 objective, 20x 0.8 NA objective, or a 63x 1.4 Oil DIC M27. For Z-stack images, step size was 5 μ m for the 5x objective, 0.5 μ m for the 20x objective and 0.3 μ m for the 63x objective. Pinhole size was 1 Airy Unit (AU) for each objective. For sagittal sections, maximum intensity projection images of Z-stack images were processed for PRG4 fluorescence intensity quantification, cell morphology analysis, and F/G-actin ratio analysis.

3.3.4 Whole Mount Imaging and Confocal Fluorescence Microscopy of Femoral Heads

For whole mount imaging, femoral heads were fixed overnight in 1mL of 4% PFA. Tissues were washed six times in PBS, femoral heads were permeabilized in 500 μ L of permeabilization/blocking buffer for 2 hours at room temperature. From here, femoral heads were stained for 2 hours at room temperature in 200 μ L of a solution containing phalloidin, DNase-I, and Hoechst. Fixed and stained femoral head cartilage was placed face down in a glass well dish with an agarose divot filled with PBS as has been done in lens whole mount imaging²⁰⁰. Super resolution Airyscan images were taken en face with the 63x objective with a zoom of 2.0 and a step size of 0.159 μ m and a pixel dwell of 2.05 μ s. For ratiometric F/G-actin analysis, femoral heads were imaged *en face* and Z-stack images (~70 stacks) were obtained of the first 20 μ m from the tissue surface. Representative single stacks were chosen for ratiometric analysis, and the cortical arrangement of actin was used to trace cell outline as ROIs. Individual cells chosen for analysis had no F-actin staining visible in their nuclei indicating that the Z-stack captured the middle of the cell. Channels were split in ImageJ and the Raw Integrated Density was taken of the red (F-actin) and green (G-actin) channels. These measurements were put in a ratio of red to green fluorescence and normalized to the

control of each experiment to determine the %F/G-actin ratio of the experimental control.

3.3.5 Image Analysis of PRG4 Quantification, Cell Circularity, Cell Area, Distance from Surface, SZC F/G-actin Ratio

PRG4 quantification was performed on maximum intensity projections of methanol fixed sagittal sections stained with PRG4, β -actin, and Hoechst. β -actin was used to define cell outline, where individual regions of interest (ROIs) were captured to represent individual cells. The fluorescence intensity of PRG4 was obtained as a function of cell area. PRG4 fluorescence was normalized to the average of intracellular PRG4 fluorescence intensities for each femoral head analyzed to allow for comparison of femoral heads despite a variation in PRG4 staining intensity (n=4). Distance from the surface was measured using ROIs which traced from the edge of the cell closest to the tissue surface to the center of PRG4 surface staining.

For cell morphology analysis, the boundaries of individual cells were manually traced using FIJI software using their cortical β -actin or F-actin arrangement to define cell outline. Cell area and circularity were calculated using FIJI software algorithms; circularity was defined as $C = 4\pi(A/P^2)$ where, P is the perimeter, and A is the cell area. A perfect circle is defined as 1; deviations from a perfect circle bring the value closer to 0. Distance from the surface was measured in μm using ROIs that traced from the closest cell edge to the surface. %F/G-actin was normalized to cells within the first 20 μm and set to 100%; cells beyond 20 μm were shown as a percentage of cells within the first 20 μm .

3.3.6 RNA Extraction, PCR, and Tpm3.1 Sequencing

For RNA extraction of cartilage from wildtype mice, femoral head cartilage tissue was scraped off the bone and immediately placed in 500 μ L of TRIzol (Sigma-Aldrich, Burlington, MA). Cartilage from 2-4 femoral heads were pooled together for each sample and crushed (~5 minutes) using a Pellet Pestle until there were no large visible pieces. Samples were stored at -20 °C until further use. Freshly isolated samples were immediately dissected; femoral head cartilage was harvested from the bone and scraped off using a scalpel under a brightfield dissection microscope in a dish filled with 1x phosphate-buffered saline (PBS; 190 GenClone).

Tpm3.1 KO mice are 50% embryonic lethal²⁰¹. To get sufficient biological repeats, we used 1 femoral head per sample for RNA extraction from experiments using Tpm3.1 KO mice. This required refining our RNA extraction protocol; isolated cartilage was scraped off bone, briefly dried with a Kim Wipe, and flash frozen in liquid nitrogen. Frozen samples were crushed using one 6 mm Zirconium bead per 2 mL tube for 60 seconds at 1000 rpm using the BeadBlaster™ 96 Ball Mill Homogenizer (#IPD9600, Benchmark Scientific, Inc., Sayreville, NJ). Samples were flash frozen again and further crushed at 1000 rpm for another 60 seconds. Samples were then placed in 400 μ L of TRIzol. To separate RNA, half the amount of chloroform was used per TRIzol amount (200 μ L or 250 μ L) for phase separation followed by selective recovery of total RNA using an RNA spin column clean-up kit (RNA Clean & Concentrator-5; Zymo, Irvine, CA). RNA was then reverse transcribed to cDNA using the UltraScript 2.0 cDNA Synthesis Kit (PCR Biosystems, Wayne, PA).

Relative real-time (RT-PCR) was performed using qPCRBio Sygreen Blue Mix (PCR Biosystems, Wayne, PA). Reactions were performed according to

manufacturer's directions; each reaction contained 20 ng of cDNA in 10 μ L of volume with primers at a 100 μ M concentration. RT-PCR reactions were run on a Cielo 3 PCR machine (Azure; Houston, TX, USA). Gene expression was normalized to relative housekeeping genes 18S or GAPDH values; the control group for each experiment was set to 100 and data from individual experiments was expressed as a percentage of the control. All PCR experiments were performed on at least 3 separate occasions with femoral head cartilage from different mice.

For semi-quantitative RT-PCR, PCR BIO Taq Mix Red solution (PCR 164 Biosystems; London, UK) was used according to manufacturer's directions on previously validated Tpm3.1 primers^{122,202}. PCR products were run on a 2% Agarose gel; resulting gel bands were excised at ~761bp and DNA was purified using GenCatch Advance Gel Extraction Kit (Epoch Life Sciences; Missouri 167 City, TX, USA). Purified DNA was sent to Genewiz for Sanger sequencing to confirm sequences (Genewiz, South Plainfield, NJ, USA).

3.3.7 Wes Capillary Electrophoresis

Protein levels in media were quantified using WES capillary electrophoresis (Protein Simple, San Jose, CA, USA) according to manufacturer's instructions, excluding dithiothreitol. 3 μ L of 500 μ L and 200 μ L of media cultured with 1 femoral head in a 48 well plate was loaded per well into a 66-440 kDa separation module plate with primary and secondary antibodies. Recombinant PRG4 and a noncommercial PRG4 antibody, 4D6, was generously gifted from Dr. Tannin Schmidt from the University of Connecticut. Media was run using Simple Western (ProteinSimple, San Jose, CA).

3.3.8 Primary Superficial Bovine Chondrocyte Isolation and Gene Expression

To obtain primary isolated superficial zone chondrocytes, we micro-dissected the metacarpophalangeal joint of cows of unknown ages. Chondrocytes of the superficial layer were dissected with a size 12 blade and placed in 0.1% collagenase and incubated overnight at 37°C. Cells were strained through an Olympus cell strainer (Genesee Scientific, San Diego, CA). Isolated chondrocytes were seeded in 500 µL of chondrogenic media (DMEM, L-proline, dexamethasone, antibiotic antimycotic) in 6 well plates at a density of 500,000 cells/mL. After 72 hours in culture, primary SZCs were treated with 3 µM of ATM-350727; cells were exposed to the treatment for 2 days then cells were harvested for RNA extraction. Media was collected and dishes were filled with 800 µL of TRIZol and left to sit for 20 minutes at room temperature prior to extraction or storage.

3.3.9 Live/Dead Assay

Femoral heads were removed from culture conditions and placed in 1mL of PBS for staining. Immediately after removal from culture, femoral heads were stained with Hoechst, Calcein Blue, and Propidium Iodide (Pi). Femoral heads were imaged at the 40x objective, and maximum intensity projections were processed. Alive cells were counted from their nuclear stain, as well as dead as denoted by staining for Pi and the percentage of alive cells was calculated.

3.3.10 Statistical Analysis

Each experiment was replicated at least three times on separate occasions. Statistical analysis and graphs were done in GraphPad Prism 9 (San Diego, CA). Outliers were detected, using the ROUT method with a 1% maximum desired false

discovery rate (Q), and were excluded from analysis. Statistical significance between two groups of data were detected using unpaired t-tests.

3.4 Results

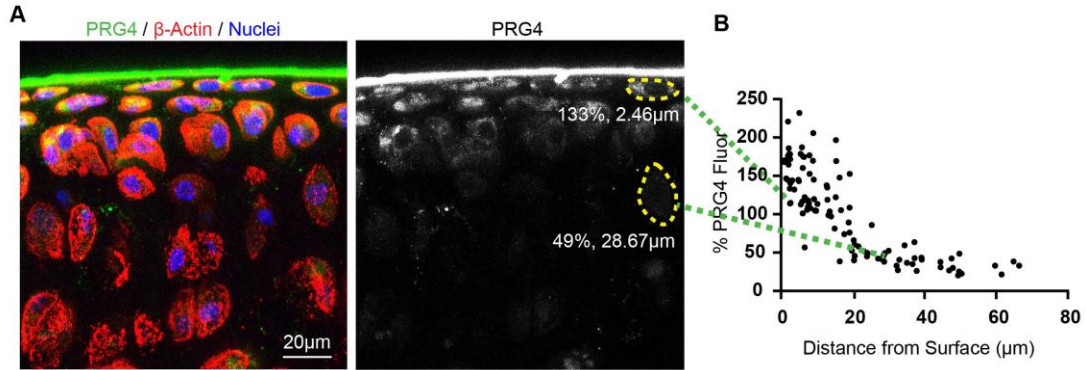


Figure 3.1 Native chondrocytes of mouse femoral head cartilage express maximal PRG4 within the first 20 microns from tissue surface. [A] Immunostaining for PRG4 shows localization at tissue surface and expression in native chondrocytes of mouse femoral head cartilage. [B] PRG4 expression is maximal within the first 20 μ m from tissue surface.

3.4.1 Mouse femoral head cartilage has a depth dependent cell morphology with unique SZCs and F-actin organization from 20 μ m of the cartilage surface

PRG4 is expressed in cells 20 μ m from the tissue surface (Figure 3.1). A hallmark feature of SZCs is the expression of PRG4²³. To primarily identify SZCs in mouse femoral head cartilage, plotted PRG4 fluorescence intensity as a function of distance from surface. We determined PRG4 to be localized at the articular surface and expressed in cells closest to the articular surface (Figure 3.1A). Cells bordering past 20 μ m from the tissue surface had substantially reduced staining for PRG4 (Figure 3.1B) suggesting that these are the resident SZCs in the mouse femoral head.

To characterize cell morphology and actin organization, we stained sagittal sections of mouse femoral head cartilage with phalloidin and DNase-I to visualize F-

and G-actin respectively (Figure 3.2A). While staining for F- and G-actin is visible throughout the tissue; the staining intensity for underlying bone is much greater than cartilage. Therefore, for image acquisition, we set the fluorescent intensity of cartilage regions within the dynamics range of our photomultiplier sensors. This led to pixel saturation of bone regions which we digitally blacked out in our images (Figure 3.S1). From these images, we show that cells closer to the tissue surface have unique cell morphology in the femoral head. We calculated cell area and circularity and plotted against distance from the tissue surface (Figure 3.2C). As compared to deeper cells, cells closer to the surface appear smaller and more elongated than cells deeper in the tissue. These cells are significantly smaller and more elongated than cells deeper (>20 μm from surface) in the tissue (Figure 3.2D) and express a significant amount more of PRG4 suggesting the residence of native SZCs 20 μm from the articular surface.

In regard to F-actin, we observed cortical F-actin organization as previously characterized in native chondrocytes³⁶ throughout the tissue (Figure 3.2B). We found depth-dependent fluorescent staining intensity for actin; cells close to the tissue surface have strong staining for F and G-actin as compared to cells from deeper zones (Figure 3.2B). Ratiometric analysis of F/G-actin shows a higher proportion of F-actin in SZCs than deeper cells (Figure 3.2E).

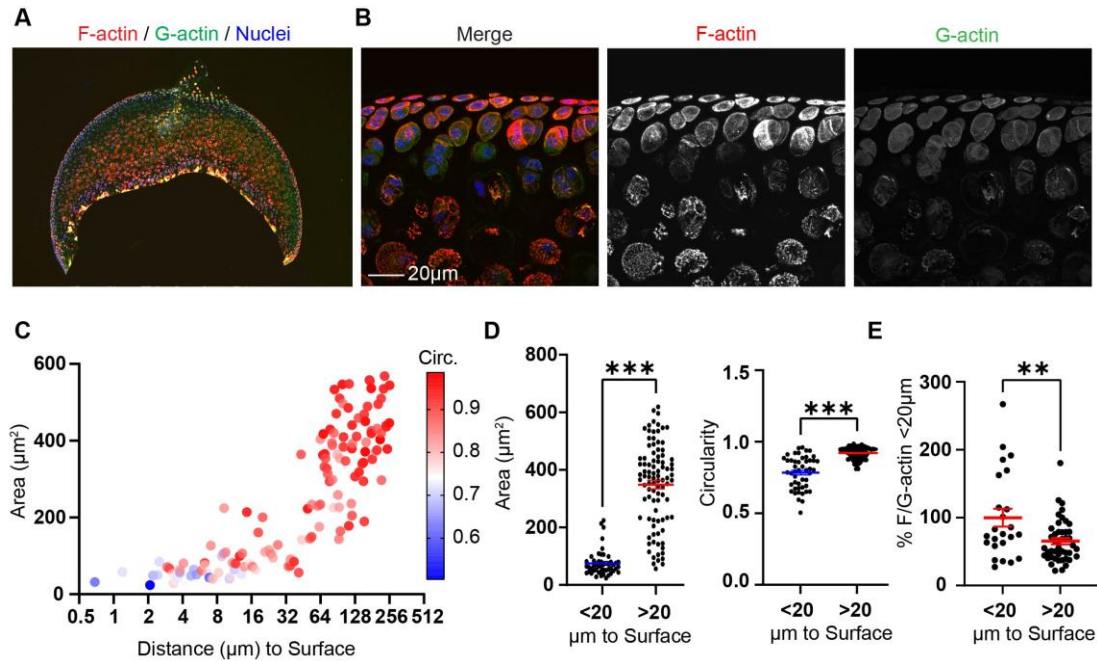


Figure 3.2 Native chondrocytes 20 μm from tissue surface have superficial zone cell morphology and unique F/G-actin. [A] The mouse femoral head has F-actin (phalloidin; red), G-actin (DNase-I; green), nuclei (Hoechst; blue), ligamentum teres (LT). Prominent staining for actin is found in the femoral head. [B] Zoomed in area denoted in magenta box shows strong staining for F- and G-actin in cells near tissue surface. [C] Area and circularity measurements plotted as distance from tissue surface, x-axis scale in $\log_2[x]$. [D] Area and circularity of native chondrocytes. [E] F/G-actin ratio is higher in SZCs ($<20 \mu\text{m}$ from tissue surface).

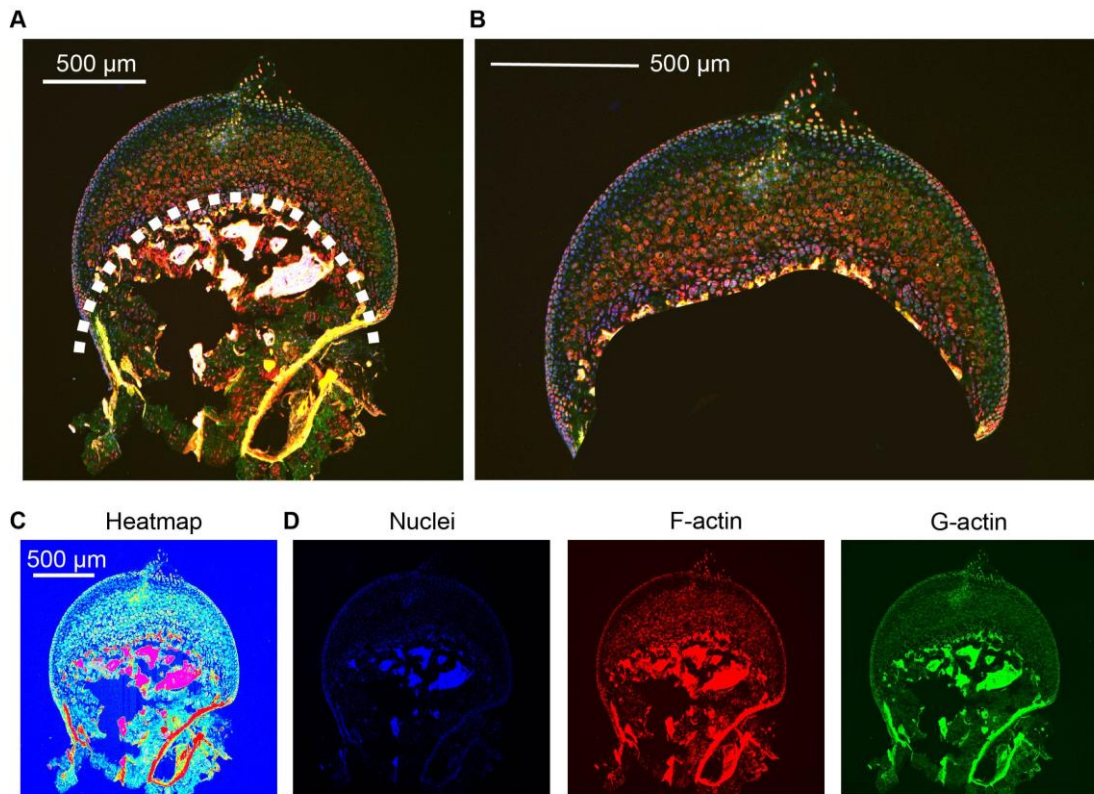


Figure 3.3 Bone regions of mouse femoral head joints have oversaturated staining compared to cartilage tissue. [A] Heat map of mouse femoral head joint shows intense staining for F-actin in bony regions greater than the cellular cartilage tissue regions. [B] Final digitally blacked out image using Photoshop.

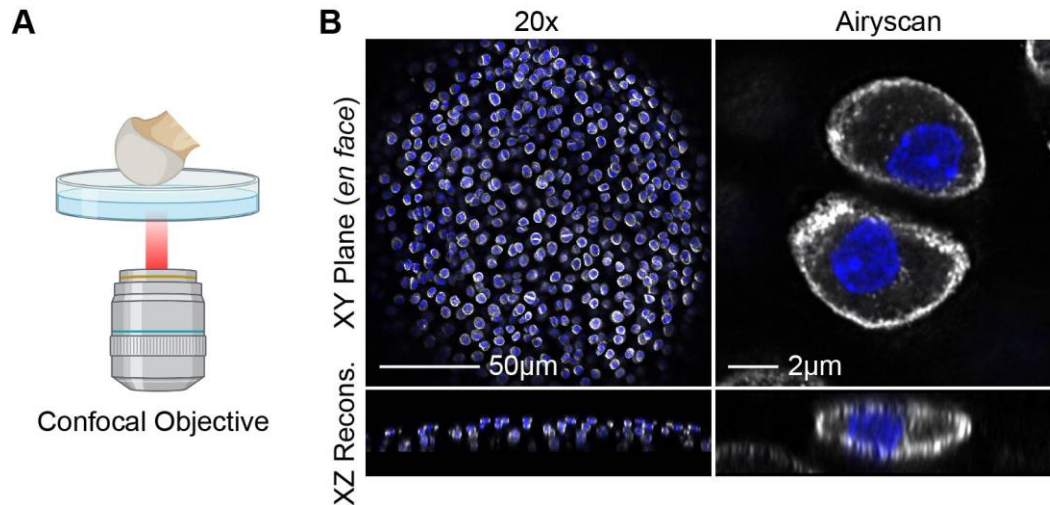


Figure 3.4 Whole mount imaging of mouse femoral head tissue identifies novel cytoplasmic F-actin networks in native superficial zone chondrocytes. [A] Confocal objective below an *ex vivo* femoral head allows for imaging of superficial zone chondrocytes up to 20 microns into the tissue. [B] En face and XZ reconstruction of Airyscan super resolution images of individual cells reveals F-actin bundles in the cytoplasm of native chondrocytes stained for F-actin (Phalloidin; gray scale) and nuclei (Hoechst; blue).

3.4.2 F-actin is not just cortically arranged in chondrocytes; native SZCs have F-actin spanning through the cytoplasm

After characterizing a population of native SCZs residing from the first 20 μm of the tissue surface, we developed a whole mount imaging protocol to visualize F-actin in native SZCs. As compared to imaging of sagittal sections, whole mount en face imaging of SZC within native cartilage provides greater spatial resolution for visualization of F-actin. Stained femoral heads were placed cartilage side down onto the confocal objective (Figure 3.4A). We were able to image approximately 30 μm into the cartilage tissue, allowing for complete imaging of SZCs. Our whole mount imaging reveals strong phalloidin staining for F-actin cortically arranged in SZCs. Additionally, Airyscan super resolution revealed prominent cytoplasmic bundles of F-

actin in SZCs. XZ reconstructions of these images provide a side view of native SZCs revealed that these F-actin bundles span throughout the cytoplasm and extend from the anterior to posterior region of the cells (Figure 3.3B). F-actin puncta are also present at the nuclear surface. These cytoplasmic F-actin bundles are relatively stable and are shown to remain despite latrunculin treatment (data not shown). F-actin is not just cortically arranged in chondrocytes and there are cytoplasmic populations of F-actin bundles in native SZCs. In addition to F-actin, we are also able to label G-actin in whole mounts by staining tissues with DNase-I (Figure 3.5). In freshly isolated femoral head cartilage, G-actin staining is faint and within the cytoplasm of SZC.

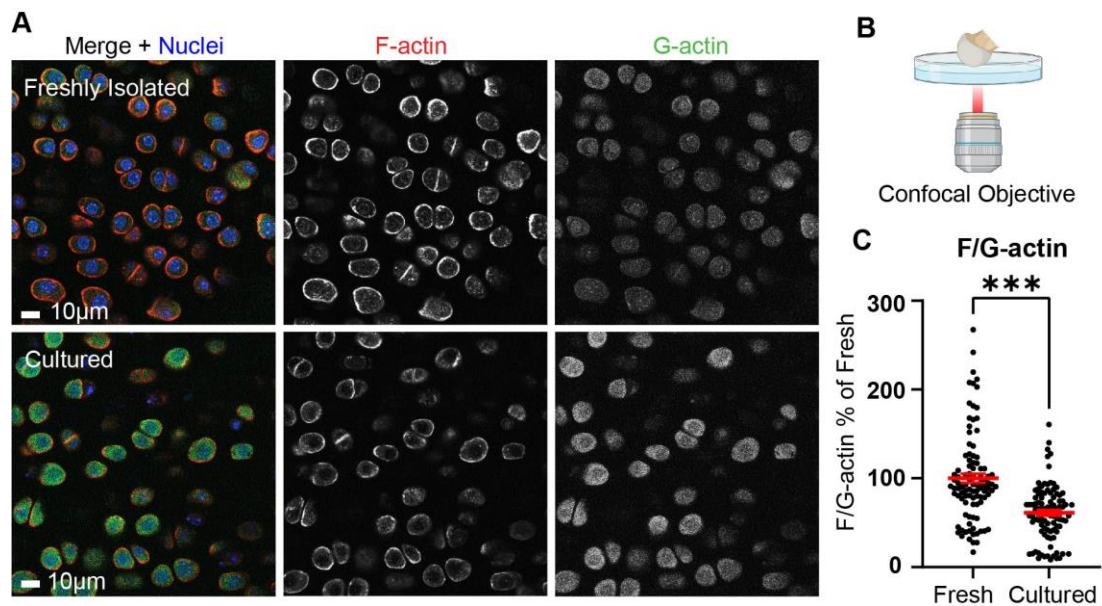


Figure 3.5 Whole mount confocal images show F-actin reorganization in superficial zone chondrocytes in response to culture conditions. [A] Femoral heads stained with F-actin (Phalloidin; red), G-actin (DNase-1; green), and nuclei (Hoechst; blue) imaged en face show bright staining for G-actin in culture conditions compared to freshly isolated (fresh). [B] Confocal objective set up for en face imaging allowing imaging of first 20 μm into tissue. [C] Fluorescence quantification of F/G-actin ratio normalized to Fresh shows decrease with culture.

3.4.3 F-actin reorganizes in native SZCs in response to culture conditions

PRG4 has been shown to be dependent on actin polymerization status *in vitro*^{32,33}, but it remains unclear if this relationship exists for F-actin in native SZCs. We previously reported that culturing isolated mouse femoral heads for up to two days recapitulates OA-like changes in gene expression including a decrease in Prg4 mRNA levels (Chapter 2). To examine the relationship between actin polymerization and Prg4 expression in native SZCs, we sought to determine if F/G-actin is altered in the native SZCs of cultured tissues.

In comparison to freshly isolated native SZCs, SZCs in femoral heads that have been cultured display reduced staining for F-actin (Figure 3.4A). Ratiometric analysis for F/G-actin in individual cells shows that culturing leads to decreased F/G-actin in native SZCs (Figure 3.4B). These results demonstrate that actin organization/polymerization status can be altered in native SZCs and that a change in polymerization status correlates with decreased Prg4 mRNA levels.

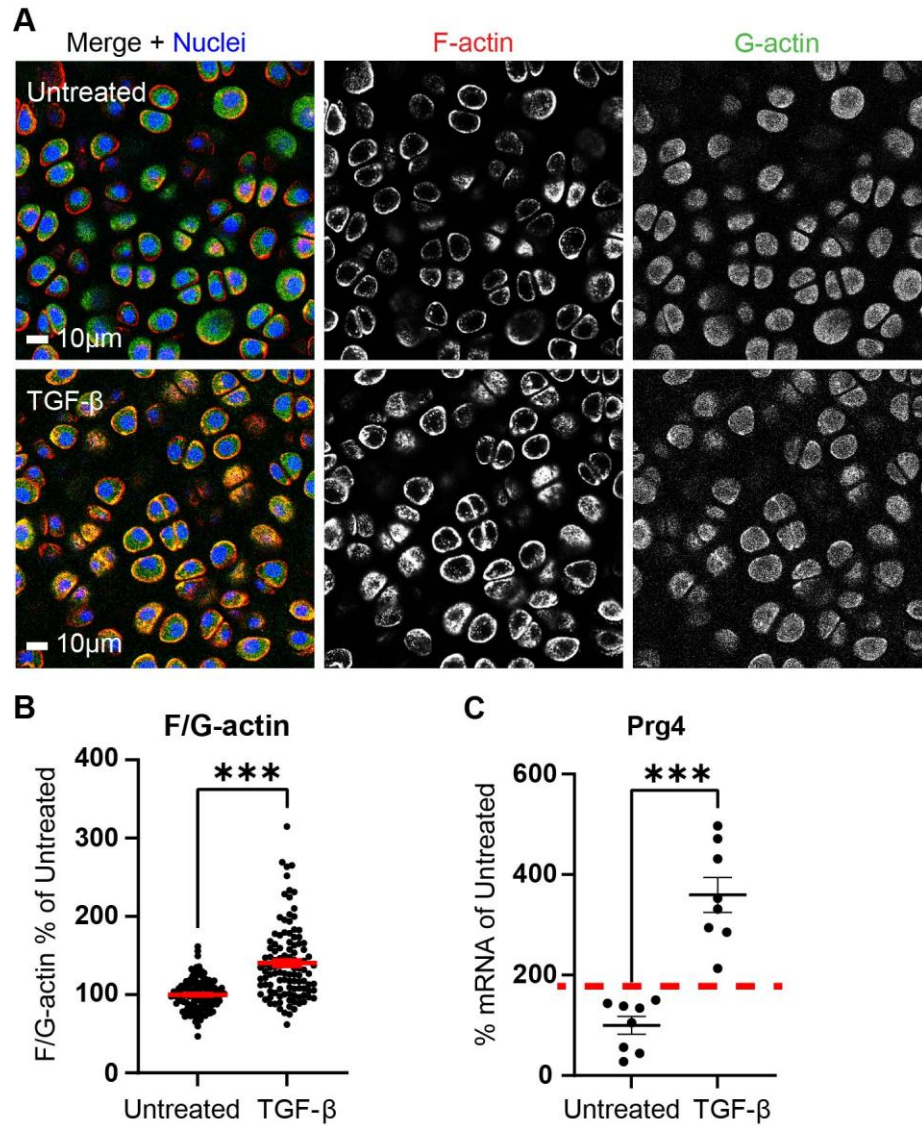


Figure 3.6 Femoral heads cultured with TGF- β show increased F-actin and Prg4 expression. [A] En face imaging of femoral heads stained for F-actin (Phalloidin; red), G-actin (DNase-1; green), and nuclei (Hoechst; blue) cultured with +/- TGF- β for 1 day. [B] The F/G-actin ratio of treatment with 20ng/ μ L of TGF- β is higher than untreated. [C] Prg4 mRNA levels increase with TGF- β treatment.

3.4.4 TGF- β stimulates F-actin and Prg4 in native superficial zone chondrocytes

Next, we sought to further alter F-actin polymerization status in native SZCs and examine Prg4 mRNA levels. TGF- β has been shown to stimulate F-actin polymerization^{58,61} and cause elongation³¹ and F-actin extensions in chondrocytes⁵⁸ as well as induce Prg4 in isolated primary SZCs^{31,54-57}. A specific study showed the induction of Prg4 was dependent on F-actin³¹. TGF- β -2 was previously reported to increase Prg4 in isolated primary bovine SZCs with treatment for 3 days⁶⁹. Hence, we tested the hypothesis that TGF- β -2 promotes elevated F/G-actin and sustains Prg4 in native SZCs of cultured femoral heads. After one day in culture, TGF- β treatment showed stronger staining for F-actin than untreated heads (Figure 3.6A). At the one-day time point the F/G-actin ratio of TGF- β treated femoral heads was higher than untreated controls (Figure 3.6B); this change in F-actin organization precedes a 4-fold increase in Prg4 at 2 days (Figure 3.6C).

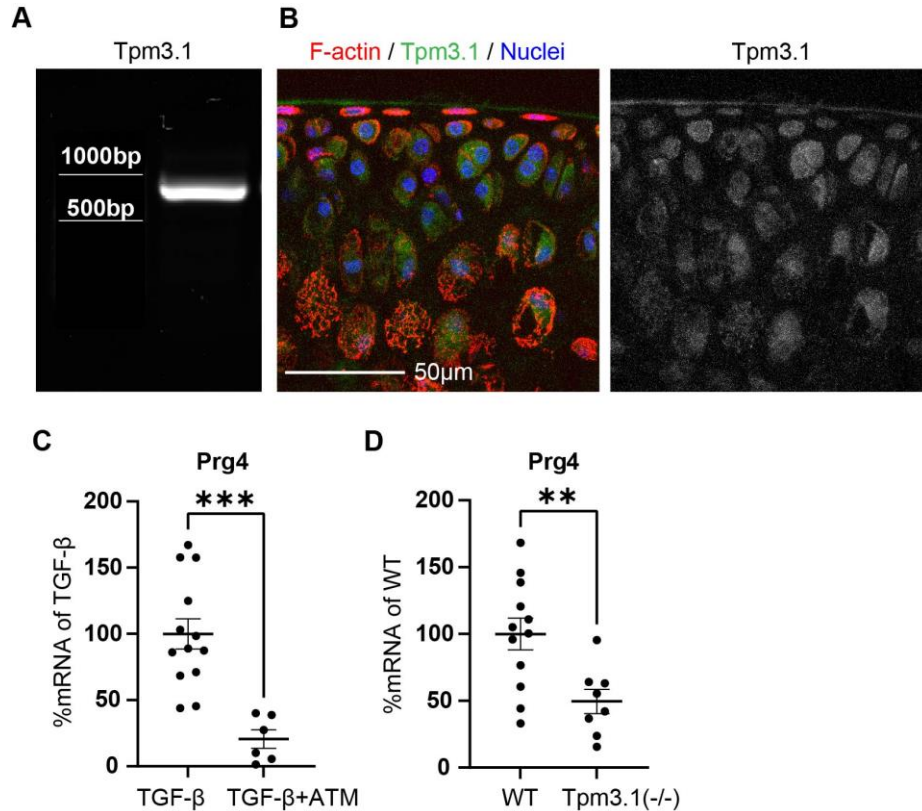


Figure 3.7 Tpm3.1 is expressed in native mouse femoral head cartilage and regulates TGF-β induced Prg4 expression. [A] Semiquantitative PCR bands from freshly isolated mouse femoral head cartilage expresses Tpm3.1 at expected ~761 bp (confirmed by Sanger Sequencing). [B] Tpm3.1 is expressed in native mouse femoral head cartilage including the superficial zone. [C] Mouse femoral heads treated with TGF-β and 4uM Tpm3.1 inhibitor ATM have decreased Prg4 mRNA levels than TGF-β treatment alone. [D] Native cartilage from Tpm3.1(-/-) mice have less induction of Prg4 via TGF-β than their wildtype counterparts.

3.4.5 Tpm3.1 is critical for induction of Prg4 mRNA by TGF-β

Since we found F-actin polymerization status and Prg4 mRNA levels to correlate, we next sought to examine if F-actin destabilization would prevent the enhancement of Prg4 mRNA levels by TGF-β. We previously demonstrated that Tpm3.1 is important for stabilizing F-actin in elongated tendon and lens epithelial

cells^{116,122}. In lens epithelial cells, inhibition of Tpm3.1 represses gene induction caused by TGF- β . Due to our findings that SZC are elongated and have enriched F-actin as compared to deeper cells, we postulated that Tpm3.1 may play an important role in stabilizing SZC F-actin. We sought to determine if inhibition or knockout of Tpm3.1 would prevent the induction of Prg4 by TGF- β .

Using PCR primers designed to detect Tpm3.1 followed by Sanger Sequencing of PCR products, we determined that Tpm3.1 is expressed in mouse femoral head cartilage (Figure 3.7A). Immunostaining for TPM3.1, using an anti-Tropomyosin 3 antibody, clone 2G10.2, shows presence of TPM3.1 in native mouse femoral head cartilage, including in SZC (Figure 3.7B). TPM3.1 localizes both with F-actin structures as well as diffuse staining within native chondrocytes which is in keeping with other cell types^{116,122,203,204}.

We next exposed femoral heads to the Tpm3.1 inhibitor, ATM-3507 which binds to Tpm3.1 to prevent its binding to other Tpm3.1 molecules, effectively reducing co-assembly with F-actin filaments¹²⁴. We determined that SZC tolerates concentrations up to 4 μ M of ATM-3507 (Figure 3.8). Tpm3.1 inhibition represses TGF- β induced Prg4 expression in mouse femoral heads (Figure 3.7C). Additionally, Tpm3.1 null mice have repressed Prg4 levels following TGF- β stimulation as compared to wildtype counterparts (Figure 3.7D).

To determine if Tpm3.1 inhibition is consistent in SZC cultured in monolayer, we isolated SZC from bovine cartilage. The bovine SZC were exposed to TGF- β either in the absence or presence of ATM3507. Treatment of primary bovine SZCs with ATM3507 selectively reduces Prg4 mRNA levels (Figure 3.9). We did not

observe alterations in other cartilage-related gene mRNA levels of collagen type II (Col2), aggrecan (Acan), or Sox9.

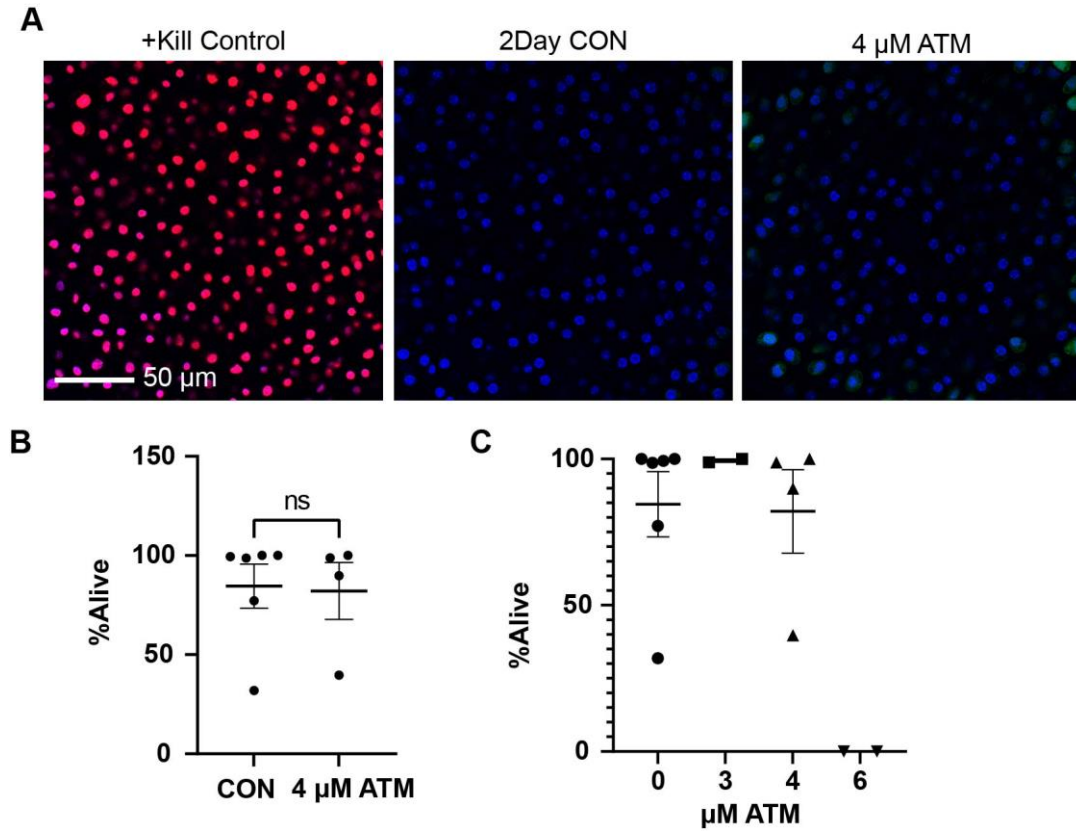


Figure 3.8 Live/Dead assay shows survival of native SZCs with up to 4 μM ATM. [A] Positive kill control with ethanol successfully kills cells while cells in femoral heads cultured for 2 days without and with 4 μM ATM have little Pi staining. [B] No significant difference between % Alive cells in femoral heads cultured for 2 days control compared to 4 μM ATM. [C] % Alive of cells shows that SZCs can withstand up to 4 μM of ATM treatment.

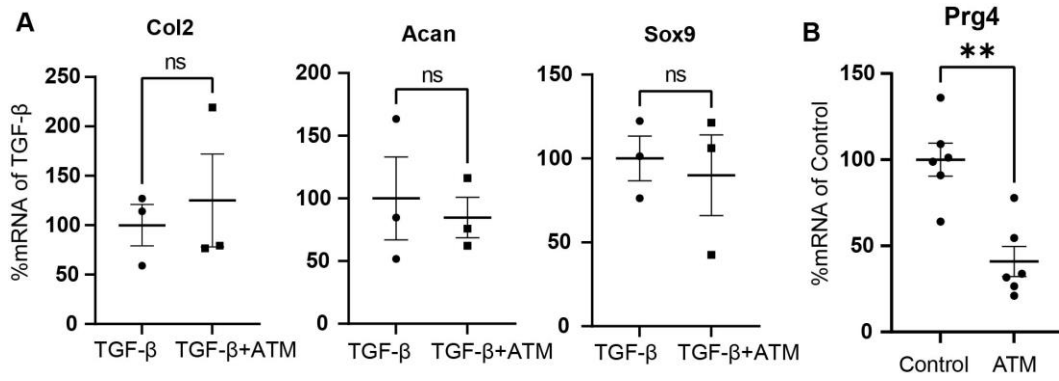


Figure 3.9 ATM treatment with TGF- β has specific effect on Prg4 and has minimal effect on other genes screened for; ATM also reduces Prg4 in primary bovine SZCs. [A] Col2, Acan, and Sox9 mRNA levels remain unaffected by addition of ATM to TGF- β treatment. [B] Prg4 decreases in primary bovine SZCs cultured with the ATM for 2 days compared to the untreated control.

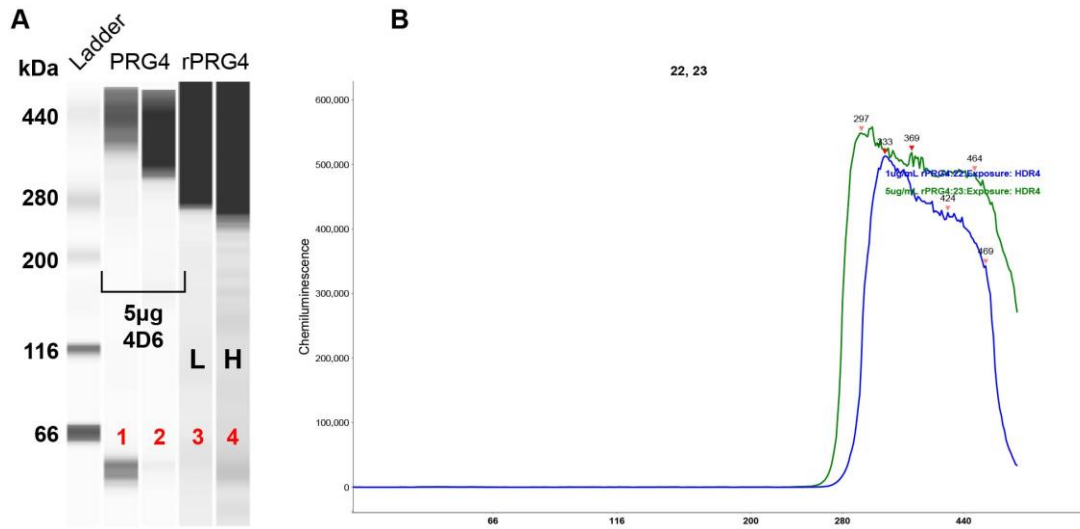


Figure 3.10 Future methodologies for quantifying PRG4 protein. [A] PRG4 protein is picked up via Wes Capillary Electrophoresis from 1 femoral head cultured with 20ng/ μ L of TGF- β in 500 μ L of media (lane 1) and 200 μ L (lane 2). Antibody 4D6 at a concentration of 5 μ g/ μ L is used to show a gradient in increased PRG4 concentration within media. Recombinant PRG4 with 4D6 antibody at 1 μ g/ μ L shows band ~430 kDa as present in mouse media [B] Peaks of Wes Capillary Electrophoresis show increased signal from 5 μ g/ μ L of recombinant PRG4 protein compared to 1 μ g/ μ L.

3.5 Discussion

In this study, we used an *ex vivo* mouse femoral head cartilage model to study the regulation of Prg4 via F-actin reorganization in the native SZCs. We provide first evidence of F-actin reorganization in native SZCs and its relationship to Prg4. Culturing reduces F/G-actin and Prg4, however these changes can be prevented by culturing femoral heads in the presence of TGF- β . We show regulation of Prg4 is dependent on Tpm3.1, a known stabilizer of F-actin.

Mouse femoral head cartilage is amenable to evaluating the relationship between native F-actin reorganization and Prg4 expression. Previous studies have used mouse knee cartilage due to the ease of destabilization of the medial meniscus (DMM)

to study articular cartilage under OA conditions, however the mouse knee is only 6-8 cell layers thick²⁰⁵ and therefore contains a minimal layer of SZCs present in this tissue. Using hip cartilage, which is round with multiple microns deep of SZCs, provides a convenient model for imaging F-actin organization in native SZCs. We characterized that mouse femoral head cartilage has a superficial zone spanning 20 μm into the tissue, which is approximately 3 cell layers thick. These SZCs are small, ovoid, and express higher PRG4 levels as compared to cells from deeper regions. Furthermore, there is enough RNA in 1 hip to perform molecular analysis; here, we developed a methodology to extract and conduct RT-PCR analysis from just 1 hip to analyze Prg4 mRNA levels. A limitation of using the femoral head is the difficulty that comes with measuring protein levels of Prg4. Since the head is so small, it secretes a dilute amount of protein into the media. Additionally, since we use native tissue, extracting protein directly from the tissue proves to be difficult due to the dense matrix. However, we have developed methodology to quantify protein levels extracted by single femoral heads cultured in 500 μL of media. Using a non-commercial antibody 4D6, we have been able to detect Prg4 in a dose sensitive manner; with low and high concentrations of Prg4 secreted by femoral heads as well as recombinant Prg4 (Figure 3.10). Future experiments will aim to test if Prg4 can be detected around the ~430 kDa weight in samples of femoral heads cultured in 1000 μL of media.

Using our whole mount imaging methodology to image F-actin in mouse femoral head cartilage, we identified a novel population of cytoplasmic F-actin bundles and demonstrated that SZC contains a pool of G-actin. While the function of cytoplasmic F-actin remains unknown, based on proximity to the nucleus, we postulate cytoplasmic F-actin bundles could play a role in transmitting mechanical

loads. However, this remains to be tested. We speculate that the maintenance of these F-actin networks as well as a maintenance of a certain pool of G-actin is critical in regulating superficial zone phenotype, including Prg4 expression, because Prg4 is stimulated mechanically by shear force^{97,99}. It remains to be tested whether mechanical stimulation of Prg4 is dependent on the cytoskeleton, however it is likely that the actin cytoskeleton is a quintessential node in chondrocyte mechanotransduction. We hypothesize that mechanical loading to SZCs due to shear stress may induce actin polymerization and provide a synergistic effect of growth factors from the synovial fluid like TGF- β . Future studies aim to delineate the role of mechanical stimulation on actin polymerization status and how this may regulate expression of Prg4 by SZCs.

Prg4 expression has been shown to depend on polymerization status to regulate the nuclear localization of myocardin-related transcription factor-A (MRTF-A); MRTF-A has a high affinity for G-actin.

To our knowledge, the current study is the first to show evidence of F-actin reorganization in native SZCs. Changes in the F/G-actin ratio over time in culture, with TGF- β treatment, and latrunculin treatment provide evidence that F-actin is capable of reorganizing in native SZCs and is dynamic. In cells with stable actin structures, latrunculin treatment has little effect due to the long turnover time of F-actin filaments; however, we have observed a decrease in F/G-actin when femoral heads were treated with latrunculin for as short as 6 hours (data not shown). Actin reorganization has been documented in isolated chondrocytes, but studies in native tissue, to this point, are limited. Studying F-actin in native tissue removes the confounds of the effect of the artificial *in vitro* monolayer environment. Future studies

aim to characterize F-actin dynamics directly using femoral head cartilage from LifeACT-GFP mice.

The high levels of F-actin have been previously characterized and speculated to be functionally necessary to withstand surface shear stress³⁶. Here, we show greater G-actin in SZC. We speculate that actin undergoes reorganization in SZCs, and a readily available pool of G-actin monomers may be required for actin remodeling.

We found Tpm3.1 to be critical in TGF- β induced Prg4. Tpm3.1 is known to associate with stress fibers in tenocytes and lens epithelial cells; we postulate that Tpm3.1 may stabilize F-actin structures that are necessary for TGF- β induction of Prg4.

We found the regulatory role of Tpm3.1 in native cartilage to be critical in TGF- β induced Prg4. Tpm3, master regulators of F-actin networks, remain vastly unstudied how they regulate chondrocyte phenotype, and as per our knowledge, it is completely unstudied what their roles are in regulating SZC phenotype. While the effect of Tpm3.1 on F-actin dynamics in native cartilage remains unknown, in lens epithelial cells and tenocytes Tpm3.1 plays a role in the stabilization of F-actin^{116,122}. We found results parallel to this as Tpm3.1 is critical for TGF β -2 induction of Prg4 in native SZCs. This is correlated with an increase in F-actin. We propose this may be stabilized by Tpm3.1 although this specific regulatory role requires further characterization. Also, it remains unclear whether there is compensation via other isoforms of Tpm3 in our knockout mouse model and how this affects Prg4 regulation. However, these results suggest that Tpm3.1 is necessary for actin modulated Prg4 expression via TGF- β in both native and *in vitro* cultured SZC. This effect is not

specific to the mouse femoral head and may be extrapolated to other joints and animal models.

In conclusion, little is known about the regulation of Prg4 in native SZCs; the elucidation of actin based signaling pathways has been conducted on *in vitro* isolated cells. Until this study, it remained to be tested if F-actin is dynamic in the SZC native environment. Our study shows regulation of Prg4 by F-actin reorganization to be dependent on Tpm3.1. An understanding of the relationship between actin and Prg4 expression in native chondrocytes could be instrumental in providing new therapeutic targets against OA progression. The superficial zone remains an important focus of study because it is the first zone to deteriorate in OA; by studying actin regulatory molecules that regulate Prg4, we may be able to elucidate an actin-based therapy for OA.

Chapter 4

DISCUSSION

In chapter 1, we discuss known regulators of Prg4 and postulate their connection with the actin cytoskeleton. Since actin pathways are dysregulated in OA and are shown to have direct connections to chondrocyte regulation, we suggest how studying the cytoskeleton in regard to Prg4 regulation may uncover molecular targets for OA cell-based therapies. Many growth factors and cytokines have been shown to modulate Prg4; we speculate some of these factors also regulate Prg4 in a cytoskeleton dependent manner that has yet to be elucidated. Additionally, some downstream regulators of Prg4 have been elucidated to be via the actin cytoskeleton, however it remains unclear if these regulate Prg4 *in vivo*. Furthermore, it remains unknown if there are key actin binding proteins that regulate actin arrangement necessary for Prg4 regulation. It may be possible to target specific actin populations in SZCs that promote Prg4 expression; understanding molecular regulators of actin regulation in SZCs may uncover druggable targets for OA therapies. We highlight a limitation in the research about actin regulation of Prg4 is that it has been only conducted in isolated chondrocytes, *in vitro*. This is limiting since actin regulation is context specific, and chondrocytes dedifferentiate in culture upon being removed from their native extracellular matrix and placed in tissue culture. Thus, to understand how actin regulates Prg4 in a more relevant context, it is critical to develop native tissue models to study this regulation.

In chapter 2, we characterize the mouse femoral head as an *ex vivo* culture model that recapitulates an OA-like phenotype. We document in native cartilage, culturing whole mouse femoral heads causes a decrease in matrix mRNA levels and an increase in catabolic and inflammatory mRNA levels. Included in these mRNA level modulations was a decrease in Prg4. We also noticed that in the stress and nutrient deprived culture system, mouse femoral head cartilage underwent an increase in G-actin proportional to F-actin. While this supports that actin can reorganize in native tissue, we show the necessity for higher resolution images to visualize F-actin arrangement. The change in actin organization correlates with a change in actin binding mRNA levels which helps support changes in actin organization and could point to regulators of F-actin in native chondrocytes.

It has been shown that actin polymerization status plays an important regulatory role in Prg4 *in vitro* and we wanted to examine this further. Thus, in chapter 3, we elucidate the role the actin cytoskeleton plays in native SZCs in Prg4 expression. Expanding on our previous culture studies, we found that culturing decreases the proportion of F/G-actin in native SZCs; this is evidence of F-actin reorganization, which correlates to a decrease in Prg4. F-actin reorganization is also evident with TGF- β treatment which increases the F/G-actin ratio; Prg4 is also stimulated with TGF- β treatment. Interestingly, we saw inhibition of TGF- β induced Prg4 mRNA levels in native SZCs via Tpm3.1 molecular inhibition or Tpm3.1 genetic knockout. This is the first study to identify the actin binding protein, Tpm3.1, as a regulator of TGF- β induced Prg4, and the first study to link actin reorganization to Prg4 expression in native SZCs (Figure 4.1). What remains undetermined is the mechanism by which Tpm3.1 interacts with F-actin to modulate Prg4 expression. We conducted preliminary

studies to visualize and quantify the F/G-actin ratio with Tpm3.1 inhibition and TGF- β treatment at various time points, however we have not seen a significant decrease. We have investigated F/G-actin at 2, 4, 8, 24, 30, and 48 hours of TGF- β treated femoral heads with molecular Tpm3.1 inhibition or in Tpm3.1 null mice versus their wildtype counterparts (data not shown). We are unable to detect a significant decrease in the F/G-actin ratio with perturbation of Tpm3.1 suggesting that either we have missed the time point or that Tpm3.1 inhibition does not perturb F-actin in a way that is detected by our ratiometric analysis. A time course experiment, observing F-actin reorganization over time may be necessary to detect a change in F-actin; this can be completed with Life-Act-GFP femoral head cartilage.

Another possibility is that Tpm3.1 may play a more specific role in stabilizing certain populations of F-actin filaments critical for TGF- β induced Prg4 that we are unable to detect using our imaging methodology. Future studies should aim to delineate where Tpm3.1 localizes to in SZCs and what role it plays in F-actin arrangement. It also may become clearer what role F-actin plays in native SZCs regulation of Prg4 if downstream regulators that have been found to be critical *in vitro*, are found in native SZCs. Overall, we have put together the first studies to our knowledge that aim at elucidating the regulatory role the actin cytoskeleton plays in native SZCs. Taken together, our study is important in supporting that the actin cytoskeleton is an important regulator of Prg4 in native SZCs and brings us one step

closer in elucidating potential druggable targets for OA therapy.

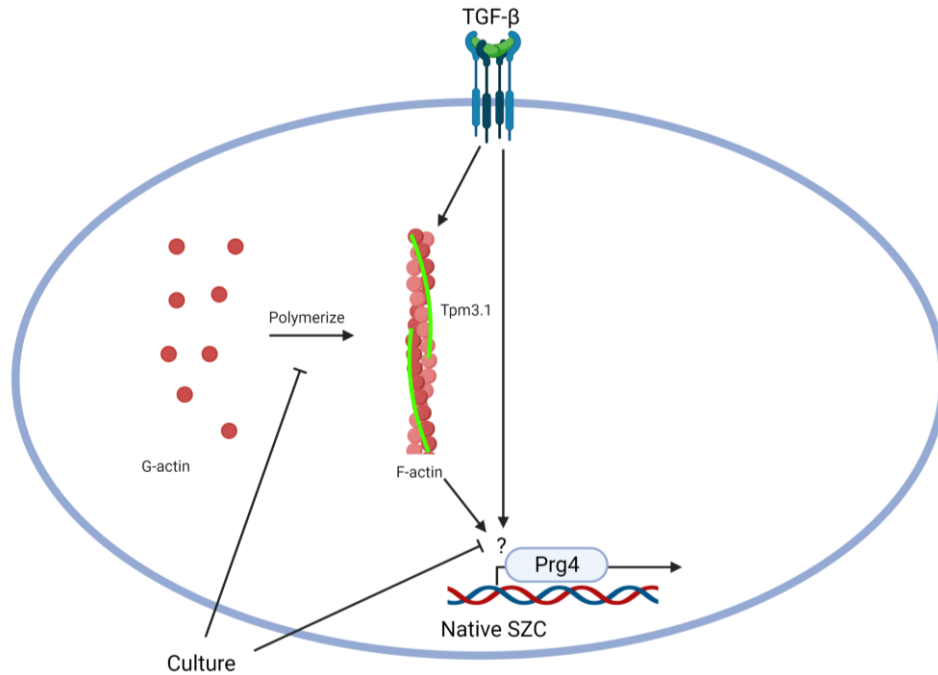


Figure 4.1 Summary of how F-actin reorganization regulates Prg4 in native SZCs. Culturing inhibits F-actin polymerization and reduces Prg4, while conversely, TGF- β stimulates F-actin and Prg4. The induction of Prg4 by TGF- β requires stabilization of F-actin with Tpm3.1. It still remains unknown what downstream regulators mediate Prg4 transcription through F-actin polymerization in native SZCs.

Chapter 5

LIMITATIONS AND FUTURE DIRECTIONS

Despite strong links between the actin cytoskeleton and regulation of chondrocyte phenotype, it remains unclear if targeting F-actin is feasible for maintaining healthy chondrocyte phenotype for OA therapies. The knowledge gaps involve understanding how F-actin reorganizes, what structures are critical for maintenance of healthy chondrocyte phenotype, and what actin binding proteins regulate the reorganization of F-actin in native chondrocytes. A long-term goal of this work is to determine key molecular regulators of F-actin networks that promote expression of Prg4 for cell based joint therapy. Throughout this exploration, our work has identified the isoform Tpm3.1 to be critical in growth factor induction of Prg4 via TGF- β . To expand these studies, we will investigate how Tpm3.1 interacts with the chondrocyte actin cytoskeleton to see if Tpm3.1 plays a role in mechanical stimulation of Prg4, and whether Tpm3.1 plays a role in the development of post-traumatic OA.

The limitation of this study thus far is that we have not confirmed if the inhibition of specifically Tpm3.1 reduces F-actin and if this is the mechanism by which it represses TGF- β induced Prg4. Nor do we know if Tpm3.1 promotes cortical F-actin architecture which may be critical to the maintenance of SZ cell F-actin networks and Prg4 expression. The future directions proposed here will provide the molecular and cellular control mechanisms by which F-actin networks are established and reorganized in chondrocytes *in vitro*, *ex vivo* and *in vivo*. The identification of

molecular regulators of F-actin networks will provide candidates to prevent OA pathogenesis.

5.1 Experiment 1: Elucidate the interaction between Tpm3.1 and the chondrocyte actin cytoskeleton via elucidating localization in different actin populations.

To complete this, we will perform Tpm3.1 and rhodamine phalloidin immunostaining on isolated primary chondrocytes. In isolated tenocytes, Tpm3.1 binds to cortical F-actin structures to stabilize elongated F-actin filaments¹¹⁶. Primarily, we have identified both cortical and novel cytoplasmic bundles of F-actin visible in native mouse SZCs. While we want to stain Tpm3.1 utilizing whole mount imaging which gives great spatial resolution, however native tissue has a dense matrix which makes antibody penetration difficult. Thus, this needs to be done in primary SZCs. Preliminary data shows that cytoplasmic bundles of F-actin are also apparent in primary isolated bovine SZCs. We aim to characterize where Tpm3.1 localizes in primary SZCs as this could give insight into the function of Tpm3.1 and what actin filament bundles are critical for Prg4 expression. Furthermore, we can test the prominence of association between actin and Tpm3.1 with TGF- β . We hypothesize that Tpm3.1 will increase in binding to F-actin with TGF- β treatment as with lens epithelial cells¹²².

5.2 Experiment 2: Elucidate downstream regulation of Prg4 via the F-actin cytoskeleton by actin related transcription factors MRTF and YAP/TAZ.

We hypothesize TGF- β induction of Prg4 is regulated by downstream transcription factors MRTF and YAP/TAZ. It has been shown in isolated SZCs that

actin polymerization status affects MRTF transcription activity due to affinity for G-actin and that Prg4 is regulated in part by this pathway³². We also hypothesize that F-actin filaments are critical in inducing mechanical stimuli to the nucleus critical for Prg4 expression via YAP/TAZ³³. To test this hypothesis, we can treat femoral heads with TGF- β as well as molecular inhibitors of MRTF, or YAP/TAZ and see if induction of Prg4 still occurs. We would expect to see a reduction in Prg4 with treatment of said inhibitors which would support the hypothesis that MRTF and YAP/TAZ regulate Prg4 in native SZCs.

5.3 Experiment 3: Characterize the function of cytoplasmic aggregates of F-actin that appear in native SZCs via perturbation of F-actin

To characterize cytoplasmic F-actin structure/stability, we can obtain regions of interest to measure their thickness/area and their angle from the longitudinal length of the nucleus in Z-stack reconstructions from Airyscan super high-resolution confocal images. Next, we can measure changes in size, thickness, and direction under different stimuli. We will image life-act-GFP femoral heads and to answer what role these filaments have; we can test their stability with latrunculin treatment/Tpm3.1 inhibition. With treatment of latrunculin, we hypothesize that these filaments have low turnover/are stable and thus would expect to see them remain unaffected with depolymerizing agents. Primary data suggests that puncta of actin remain with latrunculin treatment, while cortical actin may have a high turnover rate because these structures are affected with latrunculin treatment. We will also treat with or 3 μ M of Tpm3.1 inhibitor ATM-3507 to disrupt the stability of F-actin filaments stabilized by Tpm3.1. We hypothesize that cytoplasmic F-actin is stabilized by Tpm3.1 so these

filaments will become less prominent with ATM treatment. We can also image F-actin structures in our Tpm3.1 null mice to see differences in arrangement. To contextualize these filaments from our growth factor stimulation of Prg4, we can test if these filaments become more prominent with TGF- β treatment.

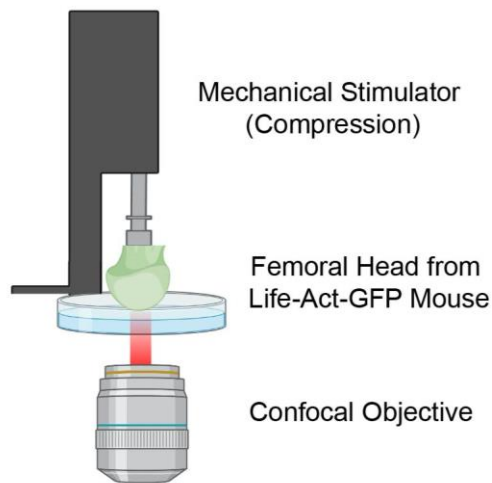


Figure 5.1 Diagram of mechanical stimulation and whole mount imaging set up. The mechanical compressor will push the femoral head with fluorescent tagged actin (live) and will be observed under the confocal objective.

5.4 Experiment 4: Contextualize cytoplasmic F-actin filaments with live imaging during mechanical stimulation.

To complete this experiment, we will apply mechanical stimulation and visualization of actin in life-act-GFP mice femoral heads. Using our imaging methodology while mechanically compressing the native tissue en face into a culture dish, we will be able to visualize the response of F-actin to mechanical stress (Figure 5.1). We will develop a system using a 3D printed construct to apply a consistent mechanical compression force to the cartilage right at the sight of where we are imaging (Figure 5.1). SZCs experiencing compressive forces will be live imaged

during a duration of 4 hours to visualize changes to the actin cytoskeleton. We will take super high resolution Airyscan images to also capture cytoplasmic F-actin aggregates. We will track changes in these filaments as well in response to mechanical loading. We hypothesize that F-actin will increase over time with mechanical stimulation, and this would correlate with increased Prg4 expression. At the end of the experiment, we can isolate the cartilage of these femoral heads and perform RT-PCR to determine relative mRNA levels of Prg4. In these studies, we will also test in our Tpm3.1 null mice if Tpm3.1 is critical for mechanical induction of Prg4.

5.5 Experiment 5: Investigate the role Tpm3.1 has in OA progression *in vivo*.

In our Tpm3.1 null mice, we can challenge these mice with unilateral destabilization of their hip joint¹⁶⁵ to model post-traumatic OA. This surgery will induce OA in one femoral head of hips in postnatal mice, to study the effect Tpm3.1 has on OA outcomes. By challenging both wildtype mice and Tpm3.1 null mice, we can compare the severity of OA outcomes using Safranin O staining and blind OARSI scoring. We predict that since Tpm3.1 is critical for growth factor induced Prg4 expression via TGF- β , and Prg4 is directly linked to beneficial outcomes in post-traumatic OA^{193,206}, this may suggest repression of Prg4 from stimuli in these joints and that Tpm3.1 null mice will experience more severe OA compared to their wildtype counterparts. If supported, this may show that Tpm3.1 is critical in protecting against OA, these mice will serve as a model of OA regulated by F-actin.

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Appendix A
IACUC APPROVAL DOCUMENT

4. Project Personnel: Have there been any personnel changes since the last IACUC approval?

Yes No

If Yes, fill out the Amendment to Add/Delete Personnel form to “Add” Personnel.

Project Personnel Deletions:

Name	Effective Date
1. Luke Mennetti	3/31/22
2. Mahbulul Shihan	3/31/22
3. Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.

5. Progress Report: If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol.

Our work aims to understand how specific actin binding proteins regulate cell shape and phenotype in normal and disease processes in tissues such as cartilage, lens, and tendon. We have been actively using mice for data collection the past year. The original protocol accurately describes all work and consequences of our work.

Since our original approval last year, we have made progress in achieving our aims (1 manuscript submitted; 2 in progress). We will continue to use mice, as indicated in our original protocol toward completion of our aims.

All mouse lines indicated in our original protocol are actively being used. Once project aims are complete and we are not actively collecting data, we will reduce animals used (decrease breeding pairs, transfer mice to other investigators, cryopreserve lines, terminate).

6. Problems or Adverse Effects: If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.


None

**University of Delaware
Institutional Animal Care and Use Committee
Annual Review**

Title of Protocol: Molecules Affecting Cytoskeleton Assembly and Downstream Actin-based Signaling	
AUP Number: 1378-2022-1	← (4 digits only)
Principal Investigator: Justin Parreno	
Common Name: Mouse	
Genus Species: Mus musculus	
Pain Category: <i>(please mark one)</i>	
USDA PAIN CATEGORY: <i>(Note change of categories from previous form)</i>	
Category	Description
<input type="checkbox"/> B	Breeding or holding where NO research is conducted
<input type="checkbox"/> C	Procedure involving momentary or no pain or distress
<input type="checkbox"/> D	Procedure where pain or distress is alleviated by appropriate means (analgesics tranquilizers, euthanasia etc.)
<input type="checkbox"/> E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation


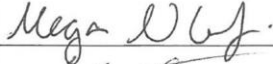

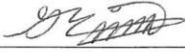
Official Use Only	
IACUC Approval Signature:	<u>Justin Parreno, DVM</u>
Date of Approval:	<u>5.1.2022</u>

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.	
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).	
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.	
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.	
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.	
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.	
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.	
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.	
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.	
10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.	
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. (<i>Teaching Protocols Exempt</i>)	
12. I understand that by signing, I agree to these assurances.	
 _____ Signature of Principal Investigator	03/29/22 _____ Date

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Justin Parreno	
2. Megan Coffin	
3. Sofia Gonzalez-Nolde	
4. Grace Emin	
5. .	
6. Click here to enter text.	
7. Click here to enter text.	
8. Click here to enter text.	
9. Click here to enter text.	
10. Click here to enter text.	
11. Click here to enter text.	
12. Click here to enter text.	
13. Click here to enter text.	
14. Click here to enter text.	
15. Click here to enter text.	

IACUC approval of animal protocols must be renewed on an annual basis.

1. Previous Approval Date: 5/18/21

Is Funding Source the same as on original, approved AUP?

Yes **No**

If no, please state Funding Source and Award Number: [Click here to enter text.](#)

2. Record of Animal Use:

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. Mouse	Mus musculus	1020 for 3 years (340 for 1 year)	246
2. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Protocol Status: *(Please indicate by check mark the status of project.)*

Request for Protocol Continuance:

A. Active: Project ongoing

B. Currently inactive: Project was initiated but is presently inactive

C. Inactive: Project never initiated but anticipated starting date is:
[Click here to enter text.](#)

Request for Protocol Termination:

D. Inactive: Project never initiated

E. Completed: No further activities with animals will be done.