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SYNOVIAL FIBROSIS IN OSTEOARTHRITIS OF THE KNEE: MECHANISM OF
DEVELOPMENT AND POTENTIAL THERAPEUTIC TARGETS

A Dissertation by

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Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Pharmaceutical Sciences

January 2021

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September 2020

**SYNOVIAL FIBROSIS IN OSTEOARTHRITIS OF THE KNEE: MECHANISM OF
DEVELOPMENT AND POTENTIAL THERAPEUTIC TARGETS**

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In dedication to the memory of my mother who is a constant source of inspiration. Dear mother, I am extremely grateful for your love, prayers, caring, and sacrifices for educating and preparing me for my future. Thanks for teaching me that the most incredible gift to be given is love! I hope I have made you proud!

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- 2- Bousoik E, **Qadri M**, Elsaid KA. CD44 Receptor Mediates Urate Crystal Phagocytosis by Macrophages and Regulates Inflammation in A Murine Peritoneal Model of Acute Gout. *Sci Rep*. 2020;10(1):5748.
- 3-**Qadri M**, Jay GD, Zhang LX, et al. Recombinant human proteoglycan-4 reduces phagocytosis of urate crystals and downstream nuclear factor kappa B and inflammasome activation and production of cytokines and chemokines in human and murine macrophages. *Arthritis Res Ther*. 2018;20(1):192.
- 4-**Qadri MM**, Jay GD, Ostrom RS, Zhang LX, Elsaid KA. cAMP attenuates TGF- β 's profibrotic responses in osteoarthritic synoviocytes: involvement of hyaluronan and PRG4. *Am J Physiol Cell Physiol*. 2018;315(3):C432-C443.
- 5-**Qadri M**, Almadani S, Jay GD, Elsaid KA. Role of CD44 in Regulating TLR2 Activation of Human Macrophages and Downstream Expression of Proinflammatory Cytokines. *J Immunol*. 2018;200(2):758-767.

ABSTRACT

SYNOVIAL FIBROSIS IN OSTEOARTHRITIS OF THE KNEE: MECHANISM OF DEVELOPMENT AND POTENTIAL THERAPEUTIC TARGETS

by Marwa Qadri

Synovial tissue fibrosis is an immune mediated disease characterized by fibroblast cell migration, proliferation and transition to a myofibroblast phenotype characterized by alpha smooth muscle actin (α -SMA) upregulation and stress fiber formation. Transforming growth factor- β (TGF- β) plays an important role in mediating the pathogenesis of synovial fibrosis. Synoviocytes produce proteoglycan-4 (PRG4; lubricin), a heavily glycosylated mucinous glycoprotein, and hyaluronic acid (HA). Both PRG4 and HA compete for binding to the CD44 receptor. Furthermore, rhPRG4 binds to TLR2 and TLR4 and acts as an antagonist to prevent TLR2 and TLR4 activation by bacterial ligands. In this work, we aimed to investigate the role of CD44 receptor and its ligands HA in regulating the activation of macrophages in response to TLR2 receptor stimulation in the context of an acute inflammatory response and study the impact of increasing intracellular cyclic adenosine monophosphate (cAMP) in OA fibroblast-like synoviocytes (OA-FLS) on regulating TGF- β linked expression of fibrotic genes and the contribution of PRG4 and HA to this effect. Also, we studied the significance of PRG4-CD44 interaction in mediating fibroblast to myofibroblast transition *in vitro* and regulation of synovial fibrosis *in vivo*. In the first manuscript, we found that anti-CD44 antibody and HA treatments reduced TLR2 induced-NF- κ B nuclear translocation, IL-1 β and TNF- α expression, and production by human macrophages. In the second

manuscript, we have shown that forskolin (10 μ M) increased intracellular cAMP levels and reduced TGF- β 1-stimulated COL1A1, α -SMA, and TIMP-1 expression in OA synoviocytes. Forskolin treatment increased HA secretion and PRG4 expression and production. Moreover, HA and PRG4 reduced α -SMA expression and content, and PRG4 reduced COL1A1 expression and procollagen I content in OA synoviocytes. In the third manuscript, we found that rhPRG4 was internalized by OA synoviocytes *via* CD44 and rhPRG4 reduced the number of stress fiber-positive myofibroblasts and cell migration in TGF- β treated NIH3T3 fibroblasts. Synovial tissues of 9-months old *Prg4*^{GT/GT} animals had higher α -SMA, collagen type-I and PLOD2 content and *Prg4* re-expression reduced these markers. In conclusion, innate immune signals trigger macrophage activation which results in synovial inflammation. Ligand-CD44 interactions reduced the extent of innate immune-mediated macrophage activation and downstream fibroblasts' response to TGF- β signals from immune cells. rhPRG4 may have potential therapeutic applications in mitigating synovitis and synovial fibrosis in advanced OA.

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LIST OF ABBREVIATIONS

8-BrcAMP	8-bromoadenosine 3',5'-cyclic monophosphate
Ab	Antibody
AC	Adenylyl cyclase
ACTA2	Actin alpha 2
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCS	Bovine calf serum
BMDMs	Bone marrow-derived macrophages
BSA	Bovine serum albumin
cADDIS	cAMP Difference Detector in situ
cAMP	Adenosine 3',5'-cyclic monophosphate
CD11b	Cluster determinant 11b
CD44	Cluster determinant 44
cDNA	Complementary DNA
CHO-M	Cho hamster ovary cells
COL1A1	Collagen type 1
CREB	cAMP response element-binding protein
Ct	Cycle threshold
CTCF	Corrected total cell fluorescence
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle Medium/Ham's F-12
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
F-actin	Filamentous actin
FA	Focal adhesion
FACS	Fluorescent Activated cell sorter
FBS	Fetal bovine serum
Fc chimeric protein	Fragment crystallizable chimeric protein
FIT-C	Fluorescein isothiocyanate
FsK	Forskolin

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTPase	Guanosine triphosphatase
HA	Hyaluronan
HAS	Hyaluronan synthase
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC	Isotype control
IgG	Immunoglobulin G
IL-1 β	Interleukin 1 beta
IL-4	Interleukin 4
I κ B	Inhibitory kappa B
kDa	kilodalton (one thousand Daltons)
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
M-PER	Mammalian protein extraction reagent
M.w.	Molecular weight
mAb	Monoclonal antibody
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	Negative control
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH3T3	NIH Swiss mouse embryo fibroblasts
OA	Osteoarthritis
OA FLS	Osteoarthritic fibroblast-like synoviocytes
Opti-MEM	Opti-minimal essential medium
PAGE	Polyacrylamide gel electrophoresis
Pam	Pam3CysSerLys4 (Pam3CSK4)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
PMA	Phorbol 12-myristate-13-acetate
PP2A	Protein phosphatase 2A
PRG4	Proteoglycan-4
PTOA	Posttraumatic osteoarthritis

qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
Raf-1	Rapidly accelerated fibrosarcoma 1
rhPRG4	Recombinant human proteoglycan-4
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640
SD	Standard deviation
SEAP	Secreted embryonic alkaline phosphatase
SF	Synovial fluids
siRNA	Small interfering RNA
Smad	Caenorhabditis elegans Sma genes and the Drosophila Mad
Src	Proto-oncogene c-Src (sarcoma)
Streptavidin-HRP	Streptavidin-horseradish peroxidase
TBS-T	Tris buffered saline-tween 20
TGF- β 1	Transforming growth factor beta 1
TIMP-1	Tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF- α	Tumor necrosis factor alpha
TRIzol	Total RNA isolation
α -SMA	Alpha smooth muscle actin

1 CHAPTER 1

INTRODUCTION

1.1 Pathogenesis of osteoarthritis (OA)

Osteoarthritis (OA) is a degenerative joint disease affecting the synovium, articular cartilage and underlying bone [1]. OA is the most prevalent arthritic disease and a leading cause of disability in the elderly [1]. OA affects approximately 37.4% of adults aged 60 years or older in the United States [2]. The prevalence of OA is on the rise and is thought to affect more than 50 million people in the United States by the year 2020 [3]. Prevalence of OA in males is lower compared with females who tend to have a severe form of OA in age 55 and older [4]. Affected joints include knees, hips, hands as well as the spine [1]. Several risk factors have been associated with OA, including genetic predisposition, advanced age, female sex, obesity and joint trauma [4].

OA is a multifactorial disease affecting the whole joint [3]. Irreversible loss of articular cartilage, inflammation of the synovial membrane, and bone osteophytes are common features of OA [5]. A complex network of risk factors, mechanical, and biochemical parameters, including cytokines and proteolytic enzymes determine the predominance of destructive processes [4, 6, 7]. Articular cartilage is a highly specialized connective tissue and is made up of extracellular matrix (ECM) (collagen and proteoglycans) and chondrocytes [8, 9]. Chondrocytes are responsible for the synthesis and breakdown of the ECM and the balance of chondrocytes' anabolic and catabolic processes is regulated by proinflammatory cytokines and growth factors [9]. Cytokines which have an impact on articular cartilage metabolism are classified in three groups including, catabolic (IL-

1 β , TNF- α), regulatory (IL-6, IL-8, IL-4, IL-10, IFN γ) and anabolic (growth factors e.g. TGF- β) [1, 10]. In OA, the balance of cartilage homeostasis is shifted towards cartilage degradation. The prevailing theory of pathogenesis in OA is that cartilage breakdown products trigger the activation of synovial macrophages causing the release of proinflammatory cytokines, like TNF- α , IL-1 and IL-6. These cytokines can bind to their chondrocyte receptors leading to further release of metalloproteinases and increase cartilage degradation [2].

The synovium is a soft tissue comprised of a surface layer, the intima, and an underlying subintima. The intima of normal synovium is one to three cell layers thick, with two cell types: fibroblast-like synoviocytes and macrophages, while the subintima includes blood and lymphatic vessels [8]. The synovium is responsible for the maintenance of synovial fluid by producing lubricin and hyaluronic acid [11]. Synovial tissue inflammation (synovitis) is a prominent feature contributing to OA progression. Histological changes that occur in synovium during synovitis include hyperplasia of the lining cell layer, infiltration of inflammatory cells e.g. lymphocytes and monocytes, and subintimal fibrosis [1, 2, 12]. Synovitis may be initiated by an innate immune response by synovial lining macrophages due to the release of damage associated molecular patterns (DAMPs) from articular cartilage and thus perpetuating the inflammation of the synovial membrane by synthesis of inflammatory mediators [1, 11] (**Fig.1.1**). Both synoviocytes and osteoarthritic chondrocytes produce large quantities of matrix metalloproteinases (MMPs), e.g. MMP-1, MMP-3, MMP-9 and MMP-13, and proinflammatory cytokines (IL-1 β , IL-6, TNF- α), which mediate disease progression and pain associated with OA [10, 13]. Accumulating evidence show that ADAMTS4 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs), is induced upon stimulation of chondrocytes and bovine cartilage explants with IL-1 β [13, 14]. ADAMTS is family of proteins that involved in cartilage degradation in OA. Clinical

investigations have revealed that patients with articular cartilage destruction had elevated MMP-13 and ADAMTS4 expressions [2]. Moreover, transgenic mice with MMP-13-overexpression show articular cartilage destruction, suggesting that increased MMP-13 may be the cause of cartilage degradation [15, 16]. Furthermore, double knockout of the ADAMTS4 and ADAMTS5 genes prevents articular cartilage erosion in mice [14, 17]. These findings indicate that MMP-13 and ADAMTS4 play a crucial role in the progression of OA, and these enzymes could be potential targets in OA treatment.

Macrophages are the most common infiltrating immune cells that are present in inflamed synovial tissue [18, 19]. Synovial macrophages play an important role in driving OA pathogenesis due to their significantly higher production of inflammatory and catabolic mediators, resulting in cartilage degeneration, synovial thickening, and osteophyte formation [19]. In addition to synovial tissue macrophages, infiltrating monocytes/macrophages may contribute to OA pathogenesis [18].

Current non-surgical treatment options focus on symptoms alleviation and these treatments don't modify the course of the underlying disease. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the mainstay for moderate to severe OA [20]. Intra-articular injections of corticosteroids for acute inflammation or hyaluronic acid for persistent OA have also been shown to be effective in patients with OA [20]. Nonetheless, no disease-modifying anti-osteoarthritis drugs (DMODs) are currently available.

1.2 Function of Proinflammatory and Anti-inflammatory cytokines in OA pathogenesis

IL-1 β is a very low molecular weight soluble peptide, produced and secreted in an inactive form, pro-IL-1 [10, 13]. IL-1 β exists in two isoforms: IL-1 α (IL-1 α) and IL-1 β (IL-1 β). IL-1 β is one of the most important proinflammatory cytokines that mediates ECM degradation and joint

inflammation in OA[10]. Its role has been identified in the late 1970s as inflammatory mediator to mimic the osteoarthritic condition on cultured chondrocytes and synovial cells [13]. Previous studies have shown that IL-1 β receptor expression was elevated in human OA chondrocytes and synovial fibroblasts compared to normal cells [10]. Binding of IL-1 β to its specific cell-surface receptor IL-1 receptor type I (IL-1RI) initiates a signaling cascade leading to the activation of transcription factors, such as nuclear factor kappa B (NF- κ B) and interferon regulatory factors (IRFs) [21]. This signaling pathway results in a variety of cellular responses including the production of pro-inflammatory cytokines and chemokines, downregulation of the synthesis of major ECM components by inhibiting anabolic activities of chondrocytes and stimulation of chondrocytes and synovial cells to release MMPs and aggrecanases [10, 13]. Other proinflammatory cytokines that are involved in OA include TNF α , IL-6 and IL-17 (**Fig.1.2**). These cytokines have been demonstrated to block chondrocytes' synthesis of type II collagen and proteoglycan and promote the production of MMPs enzymes [10].

TGF- β belongs to a large family of growth factors with an established role in controlling cell growth, proliferation, differentiation [22]. There are three isoforms of TGF- β , called β 1, β 2 and β 3 [22]. TGF- β plays an essential role in maintaining homeostasis in healthy joints. TGF- β is secreted as an inactive complex comprised of propeptide LAP (latency associated peptide) and LTBP (latent TGF- β binding proteins [22, 23]. Activated TGF- β binds to the TGF- β type II receptor to form a complex that recruits the TGF- β type I receptor, which is activated by phosphorylating serine/threonine kinase receptors [23]. TGF- β signaling is initiated with ligand-induced phosphorylation of the cytoplasmic signaling molecules Smad2 and Smad3 [23]. Phosphorylated Smad2/3 form a complex with signaling transducer Smad4 and translocate to the nucleus resulting in cell-state specific modulation of transcription [23]. TGF- β can also activate

Erk, Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways [23] (**Fig.1.3**). TGF- β 1 plays an important role in the induction of chondrogenesis and enhancing collagen synthesis [24]. Intra-articular injection of low-dose TGF- β 1 increases proteoglycan content of articular cartilage in mouse knee joints [25]. However, overexpression of TGF- β 1 in the knee joint results in OA-like changes, including hyperplasia and synovial fibrosis [26].

1.3 Role of the Toll-like Receptors (TLRs) in OA pathogenesis

TLRs are large family of receptors that belong to the family of pattern recognition receptors (PRRs) [27, 28]. There are at least 11 members of TLRs that have been identified thus far [27]. TLRs play a vital role in host defenses by recognizing pathogen-associated molecular patterns (PAMPs), including cell wall components of bacteria, viruses and fungi [29]. TLRs are also stimulated by DAMPs, and endogenous danger signal molecules that are released from injured cells [27, 29]. Examples of DAMPs that were shown to activate TLRs in the joint environment include biglycan, fibronectin and low molecular weight hyaluronan fragments, and insoluble crystals e.g. monosodium monohydrate urate and calcium phosphate crystals [30-33]. Activation of TLRs by DAMPs initiates a signaling cascade leading to the activation of transcription factors, such as NF- κ B [34, 35]. This signaling pathway results in a variety of cellular responses including the production pro-inflammatory cytokines and chemokines [35] (**Fig.1.4**). TLRs are transmembrane receptors characterized by an extracellular domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain that initiates downstream signaling [35]. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 and they mainly recognize microbial components like lipopolysaccharides [36]. Endosomal TLRs include LR3, TLR7, TLR8 and TLR9 and they recognize nucleic acids derived from bacteria and viruses [36].

The role of TLRs in OA pathogenesis has been studied. Chondrocytes derived from a TLR2/TLR4 double knockout mice resisted the pro-catabolic effect of low molecular weight hyaluronan and showed attenuated MMP-13 expression [37]. Furthermore, TLR2 and TLR4 can act synergistically to increase inflammatory cytokine production in OA synovial cells [38]. Early reports showed that synovial fluid (SF) from patients with early OA augments synoviocytes responses to TLR2 and TLR4-ligands and that plasma proteins present in OA SF stimulate cytokine production in a TLR4-dependent manner [39]. Additionally, it has been recently observed that OA SF activates TLR2 and TLR4 on human embryonic kidney cells [40].

Moreover, the role of innate immune system in mediating tissue remodeling and fibrosis has been highlighted in a number of studies. Recent studies showed that activation of TLR4 sensitizes skin fibroblasts to the stimulatory effect of TGF- β 1 and enhances the accumulation of ECM molecules [41]. Moreover, fibroblasts isolated from *TLR4*^{-/-} mice showed attenuated profibrotic activities of TGF- β 1, suggesting the innate immune signaling via TLRs are essential for fibroblast activation [41]. Interestingly, synovial resident cells have been shown to express TLRs and persistent inflammation might play a considerable role in the development of synovial fibrosis.

1.4 Synovial fibrosis in advanced OA

Major abnormalities observed in the OA synovium include synovial hyperplasia, inflammatory cell infiltration, angiogenesis and fibrosis [11]. Fibrosis is a non-physiological wound healing process that is characterized by excessive deposition of ECM proteins, in particular collagens coupled with reduced turnover [42]. Synovial fibrosis is a common feature in advanced OA that contributes to joint pain and stiffness [42]. Synovial fibrosis is a prevalent outcome of total knee arthroplasty (TKA) where the collagen-rich dense fibrous tissue limits the normal range of motion

and contributes to functional deficits [43]. Patients with synovial fibrosis are more likely to experience a first and follow-up TKA and reducing the rate of fibrosis may reduce the likelihood of a revision TKA [44]. The underlying mechanism that causes synovial fibrosis has not been investigated.

The TGF- β signaling pathways play an essential role in maintaining joint homeostasis in healthy joints, and a switch to a pathologic role that drives synovial fibrosis [45]. Blaney *et al* showed that intra-articular injection of adenovirus overexpressing active TGF- β in the knee joints resulted in a significant increase of synovial thickness 2 weeks after injection [46]. Additionally, previous observations showed that TGF- β upregulates the expression of synovial collagen type 1, tissue inhibitor of metalloproteinase 1 (TIMP-1), and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) and promotes the differentiation of OA synovial fibroblasts into a myofibroblast-like phenotype [47]. Myofibroblasts have enhanced ability to express alpha smooth muscle actin (α -SMA) and produce collagen, proliferate and migrate [47, 48].

Synovial macrophages play an important role in driving OA pathogenesis due to their significantly higher production of inflammatory and catabolic mediators, resulting in cartilage degeneration, synovial thickening, and osteophyte formation [19]. In addition, local depletion of synovial macrophages was shown to mitigate cartilage damage and osteophyte formation in experimental OA models [49]. Using a macrophage Fas-induced apoptosis (MaFIA) transgenic mouse, clodronate liposomes-mediated depletion of synovial lining macrophages was shown to limit cartilage damage and synovial inflammation [49]. In addition to synovial tissue macrophages, infiltrating monocytes/macrophages may contribute to OA pathogenesis [18]. Monocytes and macrophages are found with increased numbers in arthrofibrotic tissues, which may signal their

involvement in the pathogenesis of synovial fibrosis [50]. Furthermore, chemotactic factors *e.g.* IL-8 and MCP-1 were highly expressed in fibrotic synovial tissues, infrapatellar fat pad, and SF from patients with revision TKA compared to patients with non-fibrotic primary TKA [43, 50, 51].

Moreover, inflammatory microenvironment in OA synovium has been implicated in switching both synovial-resident and infiltrating monocytes/macrophages into two main subsets M1 (pro-inflammatory) to M2 (anti-inflammatory) [52]. Following tissue injury, monocytes migrate and differentiate into M1 inflammatory macrophages [53]. Activation of M1 macrophages is achieved by inflammatory cytokines *e.g.* IFN- γ and via the toll-like receptor system [52, 54]. M1 macrophages produce TGF- β as a regulatory feedback signal to aid in the resolution of inflammation [54, 55]. One of the first steps following tissue injury is fibroblasts migration, differentiation into myofibroblasts and production of matrix proteins in response to TGF- β [55, 56]. M2 macrophages are anti-inflammatory by virtue of producing IL-10 and TGF- β , and in this regard antagonize the profibrotic role of M1 macrophages [57]. Excessive M2 macrophage responses can contribute to pathologic fibrosis via increased production of profibrotic mediators *e.g.* TGF- β [57, 58]. Our understanding of the contribution of M1 and M2 macrophages to OA synovitis and synovial fibrosis remains limited. Available evidence supports that synovial macrophage M1 polarization exacerbated synovitis and resolution of synovial inflammation was associated with the population of the synovium by M2 macrophages that possess a homeostatic anti-inflammatory phenotype [59, 60]. Furthermore, analysis of aspirates collected from patients with advanced OA shows that the concentrations of CD163 and CD14, M2 macrophage soluble markers, were higher in synovial fluid and plasma in these patients [61]. These data suggest that monocytes/macrophages may play a role in regulating synovitis and ensuing synovial fibrosis.

1.5 Cyclic adenosine monophosphate

Cyclic adenosine monophosphate is a pleiotropic intracellular second messenger generated by adenylyl cyclase enzymes in response to G-protein coupled receptor (GPCR) activation [62]. The antifibrotic effect of cAMP has been shown in fibroblasts from multiple origins and includes inhibition of fibroblast proliferation, fibroblast-myofibroblast transition, reduction in fibroblast migration and synthesis of ECM components [63, 64]. It has been shown that isoproterenol, a cAMP-elevating agent, inhibited TGF- β -stimulated collagen synthesis and α -SMA expression in cardiac fibroblasts [63]. Also, stimulation of cAMP production blunted TGF- β -induced pulmonary fibroblasts proliferation [64]. The increase in cAMP results in activation of protein kinase A (PKA)-dependent and -independent pathways [65, 66]. In the presence of growth factors, cAMP elevation activates cAMP-dependent PKA, which interferes with Raf-1 activation and signaling to blunt cell proliferation [67]. PKA also activates CREB, which can compete for cofactors with SMAD-mediated transcription stimulated by TGF- β 1 [63]. In the absence of growth factors, PKA-dependent and independent pathways stimulate cAMP-mediated cell proliferation [68]. However, the role of cAMP in controlling TGF- β fibrotic response in osteoarthritic synovial fibroblasts remains unknown.

1.6 Homeostatic roles of proteoglycan-4 (PRG4) and hyaluronan in the articular joint

Lubricin/Proteoglycan 4 (PRG4) is a glycoprotein secreted from synovial fibroblasts and superficial zone chondrocytes and has a multifaceted function including boundary lubrication, friction lowering of apposed cartilage surfaces and prevention of synovial overgrowth [69-72]. PRG4 is abundant in synovial fluid (SF) and PRG4 SF levels are reduced following acute joint trauma and in patients with osteoarthritis and rheumatoid arthritis (RA) [73, 74]. In animal models

of posttraumatic osteoarthritis (PTOA) and inflammatory arthritis, *Prg4* expression is reduced in cartilage and synovium [75-77]. Cartilage biosynthesis of PRG4 is reduced as a result of IL-1 β and TNF α exposure but is increased with TGF β [78, 79]. In synoviocytes, *Prg4* expression is reduced by IL-1 β and is increased by TGF β and TGF β -linked PRG4 accumulation is counterbalanced by IL-1 β [79, 80]. Loss of function mutations in the *Prg4* gene is evident in the autosomal recessive disease Camptodactyl-Arthropathy-Coxa Vara Pericarditis (CACP) syndrome which is a juvenile-onset arthropathy [81]. This phenotype is recapitulated in the *Prg4* knockout (*Prg4*^{-/-}) mouse, which displays progressive synovial hyperplasia, cartilage surface fibrillations and damage [81]. PRG4's joint homeostatic role is further evidenced by the ability of purified human PRG4, recombinant full-length or truncated PRG4 to retard cartilage degeneration, enhance cartilage repair and reduce chondrocyte apoptosis in PTOA models [82]. PRG4's protein core is 1,404 amino acids long with globular N and C termini and a central mucin domain (**Fig. 1.5**). The central mucin domain is heavily glycosylated via O-linked β 1-3)Gal-GalNAc oligosaccharides, and is configured to form a nanofilm that exerts repulsive forces, and provides the basis for its anti-adhesive and lubricating properties [83]. Removal of the saccharides from PRG4 results in a loss of boundary lubricating ability, while the C-terminus may facilitate surface binding of the protein [83]. The specific pattern of glycosylation may play a role in mediating PRG4's biological functions [84]. PRG4 from RA SF exhibits increased sialylation potentially leading to an altered glycosylation-dependent biological role for PRG4 [85]. PRG4 is shown to bind to L-selectin in a glycosylation-dependent manner and coats polymorphonuclear granulocytes recruited to inflamed synovia and SF of patients with RA [84, 85]. PRG4 was shown to bind the CD44 receptor in a concentration-dependent manner and competes with high-molecular weight hyaluronan, a CD44 ligand, on binding to CD44 [86]. rhPRG4-CD44 interaction mediates

rhPRG4's ability to inhibit IL-1 β and TNF α -induced proliferation of fibroblast-like synoviocytes from patients with RA. The downstream effect of engaging the CD44 receptor by rhPRG4 is blocking NF- κ B nuclear translocation [86]. Also PRG4 was shown to bind TLR2 and TLR4 receptors and acts as an antagonist to prevent TLR2 and TLR4 activation by bacterial ligands [40].

HA, a major ECM glycosaminoglycan, is a high molecular-weight polysaccharide [87]. HA is produced by fibroblast-like synoviocytes and synthesized as high-molecular weight (HMW) forms (MW, >1000 kDa) by HA synthase (HAS) with three isoforms identified to date (HAS1, HAS2, and HAS3) [88]. HA is a primary lubricant molecule in synovial fluid with concentration ranges 1 to 4 mg/mL [87]. HA mediates its biological functions through binding to transmembrane receptor, CD44 [89]. Recent studies showed that high molecular weight hyaluronic acid (HMW-HA) suppressed IL-1 β –mediated NF- κ B activation and downstream expression of MMP-1 and MMP-3 in OA synovial cells in a CD44-dependent manner [90]. Additionally, HA's anti-inflammatory effects may be mediated by its ability to modulate TLR2 and TLR4 cartilage expression in experimental arthritis [91]. In addition, HA play an important role in joints fibrosis, as intraarticular injection of HA reduced TGF- β 1-induced synovial fibrosis in a murine model of OA [92].

1.7 Cluster determinant 44 (CD44) receptor and associated signaling pathways

CD44 is a transmembrane glycoprotein, with various isoforms generated by alternative splicing, plays a crucial role in cell adhesion, development, endocytosis, tissues homeostasis, and in a variety of inflammatory diseases [93]. CD44 has an established role in hyaluronan metabolism and cellular uptake [89, 93]. CD44 was shown to bind to osteopontin, collagen, and MMPs and different ECM components including PRG4, HA [94].

Previous studies have suggested that CD44 has role in OA development. CD44 expression was detected in synovia and cartilage of patients with different severities of OA [95]. CD44 receptor has a role in regulating cell signaling pathways. A study by Rothman *et al* demonstrated that anti-CD44 mAb elevates cAMP and inhibits the increase in intracellular Ca^{2+} in human T lymphocytes [96]. It was suggested that CD44 might be positively coupled with adenylyl cyclase and that activation of adenylyl cyclase by anti-CD44 mAb or forskolin, an adenylyl cyclase activator, may inhibit the increase in intracellular Ca^{2+} in human T lymphocytes [96]. In the context of innate immunity, CD44 was found to regulate Fc γ and complement receptor 3-dependent macrophage phagocytosis [93, 97]. Additionally, CD44 may play a role in the negative regulation of TLR receptor activation [98]. Furthermore, CD44 engagement by the antibody resulted in PP2A activity activation and inhibition of PP2A activity reversed the anti-inflammatory effect of the antibody [99]. In addition to its role in mediating cellular migration and inflammation, CD44 receptor contributes to fibrosis in response to injury. *CD44*^{+/+} fibroblasts produce more active TGF- β 1 than *CD44*^{-/-} fibroblasts [100], suggesting an effect of CD44 targeting in suppressing the activation of fibroblasts.

1.8 Significance

OA fibrosis is a common outcome of advanced OA that contributes to joint pain, stiffness and functional deficits. OA fibrosis is an immune mediated disease characterized by fibroblast cell migration, proliferation and transition to a myofibroblast phenotype characterized by alpha smooth muscle actin (α -SMA) upregulation and stress fiber formation. Synoviocytes produce proteoglycan-4 (PRG4; lubricin), a heavily glycosylated mucinous glycoprotein, and hyaluronic acid (HA). PRG4 is abundant in synovial fluid (SF) and PRG4 SF levels are reduced following

acute joint trauma and in patients with OA. PRG4 play an important role in joint lubrication and it binds to CD44, the HA receptor, to exert an anti-inflammatory effect in the articular joint. The role of synovial PRG4 in the context of resolution of synovitis and development of fibrosis remains unknown. Moreover, the pathological mechanisms that cause OA fibrosis are unclear, and current treatments include physical therapy and revision surgeries. Given that OA fibrosis develops in ~3-10% of patients with total knee arthroplasty (TKA), there is a significant need for new prophylactic and/or treatment modalities. This study expands our understanding of the pathophysiology of OA fibrosis and provides us with the rationale to pursue PRG4 as a promising biologic treatment to prevent and/or treat OA fibrosis.

1.9 Hypothesis

PRG4 in synovial fluid possesses an anti-inflammatory and antifibrotic effect that prevents immune-mediated TGF- β activation and OA fibrosis is associated with a reduction in PRG4's biological effect.

1.10 Specific Aims:

We propose *three interconnected* aims to test our overall hypothesis:

Aim 1: To evaluate the role of CD44 in regulating NF- κ B activation and proinflammatory cytokine production in response to TLR2 receptor activation in human macrophages using a combination of CD44 receptor knockdown, TNF- α production by macrophages from CD44 wildtype and knockout mice, and CD44 receptor engagement by CD44-neutralizing antibody and HA treatments.

Hypothesis:

CD44 regulates TLR2 activation of human macrophages, whereby a reduction in CD44 levels or engagement of CD44 by its ligand (HA) or CD44-specific antibody reduces downstream pro-inflammatory cytokine production.

Aim 2: To investigate the antifibrotic function of cAMP, generated by forskolin treatment, in TGF- β stimulated OA synoviocytes and study the contribution of synovial fluid components PRG4 and HA to OA fibrosis.

Hypothesis: Increasing intracellular cAMP exerts an antifibrotic effect in OA synovial fibroblasts and promotes PRG4 and HA secretion.

Aim 3: To examine the role of PRG4-CD44 interaction in regulating synovial tissue fibrosis *in vitro* and *in vivo*.

Hypothesis: PRG4 regulates fibroblast to myofibroblast transition and modulates fibroblast migration in response to exogenous TGF- β or co-incubation with lipopolysaccharide (LPS) stimulated macrophages in a CD44-dependent manner.

1.11 Figures

Figure 1.1 Involvement of the synovium in OA pathophysiology. Products of cartilage breakdown that are released into the synovial fluid are phagocytosed by synovial cells, amplifying synovial inflammation [12].

Figure 1.2 The role of proinflammatory cytokines in the pathophysiology of osteoarthritis (OA). The levels of proinflammatory cytokines, including IL-1 β , TNF and IL-6, are elevated in OA [10]

Figure 1.3 Canonical and non-canonical TGF- β signaling pathways. (A) In the canonical signaling pathway, biologically active TGF- β ligands bind to TGF β RII, which in turn activates TGF β RI. TGF β RI-regulated SMAD2/3 proteins are phosphorylated at their C-terminal serine residues and form complexes with SMAD4 (co-SMAD), initiating a number of biological processes through transcriptional regulation of target genes. (B) In the non-canonical signaling pathways, the TGF- β receptor complex transmits its signal through other factors, such as the mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), and TNF receptor-associated factor 4/6 (TRAF4/6). Activated MAPKs can exert transcriptional regulation either through direct interaction with the nuclear SMAD protein complex or via other downstream proteins. Moreover, TGF- β activation of the TRAF proteins can initiate nuclear factor- κ B (NF- κ B) signaling activity, leading to the inflammatory response among other processes [101].

Figure 1.4 Toll-like receptor signaling pathway. Toll-like receptors (TLRs; with the exception of TLR3) induce nuclear factor- κ B (NF- κ B) dependent cytokine production through a pathway involving the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88). However, TLR3 (and TLR4) transduce signals through a MyD88-independent signaling pathway that involves the adaptor molecule Toll/IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF) [102].

Figure 1.5 Schematic depicting the various motifs within the 1,404 amino acid proteoglycan-4 (PRG4) polypeptide.

2 CHAPTER 2

Role of CD44 in Regulating Toll-like Receptor 2 (TLR2) Activation of Human Macrophages and Downstream Expression of Proinflammatory Cytokines

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Abstract

Osteoarthritis (OA) is a low-grade chronic inflammatory joint disease. Innate immunity contributes to OA progression, mediated by toll-like receptors (TLR2 and TLR4). We evaluated the role of CD44, a transmembrane glycoprotein, in regulating TLR2-linked macrophage activation and resultant proinflammatory responses. TLR2 stimulation was performed on differentiated THP-1 macrophages in the presence or absence of a CD44-specific antibody or hyaluronan (HA). Nuclear factor kappa B (NFκB) nuclear translocation; interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) gene expression and protein concentrations were determined. Anti-CD44 antibody and HA treatments reduced NFκB translocation, IL-1β and TNF-α expression and production ($p<0.001$). Inhibition of proinflammatory response in macrophages by HA was mediated by CD44. Protein phosphatase 2A (PP2A) mediated the reduction in NFκB translocation by HA. CD44 Knockdown reduced NFκB nuclear translocation and downstream IL-1β and TNF-α protein production following TLR2 receptor stimulation ($p<0.001$). *CD44^{+/+}* murine bone marrow derived macrophages (BMDMs) produced higher TNF-α compared to *CD44^{-/-}* macrophages following TLR2 stimulation ($p<0.01$). HA dose-dependently inhibited TLR2 induced TNF-α production by murine BMDMs ($p<0.001$). OA synovial fluids (SF) stimulated TLR2 and TLR4 receptors and induced NFκB translocation in THP-1 macrophages. Anti-CD44 antibody treatment significantly reduced macrophage activation by OA SF ($p<0.01$). CD44 regulated TLR2 responses in human macrophages, whereby a reduction in CD44 levels or engagement of CD44 by its ligand (HA) or a CD44-specific antibody reduced NFκB translocation and downstream proinflammatory cytokine production. A CD44-specific

antibody reduced macrophage activation by OA synovial fluids and CD44 is a potentially novel target in OA treatment.

2.1 Introduction

Osteoarthritis (OA) is a degenerative joint disease involving joint structures *e.g.* the articular cartilage, synovium and subchondral bone [103, 104]. Risk factors for OA pathogenesis include age, obesity, traumatic joint injury, and low-grade chronic inflammation [103, 105-109]. Inflammation of the synovial tissue is recognized as an active contributor to OA pathogenesis with multiple studies showing a correlation between the extent of synovitis and pain, cartilage erosion and disease progression [110-114]. The synovium is a soft tissue that is comprised of a surface layer, the intima and an underlying subintima [112, 115]. The intima of normal synovium is 1-3 cell layers thick, with two cell types: fibroblast-like synoviocytes and macrophages [115, 116]. Chronically inflamed synovium, as seen in OA, is characterized by synovial intimal hyperplasia, immune cell infiltration, subintimal fibrosis and neovascularization [117, 118].

Infiltrating immune cells detected most frequently in OA synovial tissues include macrophages, T cells and to a lesser extent mast cells and B cells [117, 119, 120]. Synovial macrophages play an important role in driving OA pathogenesis due to their significantly higher production of proinflammatory cytokines *e.g.* interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) compared to fibroblast-like synoviocytes [121, 122]. Activation of synovial macrophages was shown to promote synovial thickening, osteophyte formation and cartilage degeneration in experimental OA models [123, 124]. As a component of the innate immune system, macrophages can be activated by damage-associated molecular patterns (DAMPs) through interaction with pattern recognition receptors, *e.g.* toll-like receptors (TLRs) on the surface of macrophages [125]. Examples of DAMPs in OA include extracellular matrix breakdown products *e.g.* biglycan, fibronectin fragments, low molecular weight hyaluronic acid, plasma proteins *e.g.* α 1 and α 2

macroglobulins, intracellular alarmins *e.g.* high mobility group box 1 (HMGB1) and crystals *e.g.* uric acid [126].

Hyaluronan (HA) is produced by fibroblast-like synoviocytes and is a major component of the synovial fluid. HA exerts important functions in the joint and its biological effects are mediated by its binding to its transmembrane receptor, cluster determinant 44 (CD44) [127]. In OA synoviocytes, HA suppresses IL-1 β mediated nuclear factor kappa B (NF κ B) activation and downstream expression of matrix metalloproteinases (MMPs) in a CD44-dependent manner [128, 129]. Additionally, HA's anti-inflammatory effects may be mediated by its ability to modulate TLR2 and TLR4 cartilage expression in experimental arthritis [130].

In this work, we aimed to evaluate the role of CD44 in regulating NF κ B activation and proinflammatory cytokine production in response to TLR2 receptor activation in human macrophages, using a combination of CD44 receptor knockdown, TNF- α production by macrophages from CD44 wildtype and knockout mice, and CD44 receptor engagement by CD44 neutralizing antibody and HA treatments. We hypothesized that CD44 functions to regulate NF κ B activation and downstream IL-1 β and TNF- α gene expression and production in response to TLR2 stimulation of a human macrophage cell line. Finally, we studied TLR2 and TLR4 receptor activation by OA synovial fluids and examined the effect of CD44 targeting in suppressing the activation of macrophages by these fluids.

2.2 Materials and Methods

2.2.1 Differentiation of THP-1 monocytes into macrophages

THP-1 monocytes were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured to a density of 1.5×10^6 cells/mL in 75 cm flask in RPMI 1640 medium

supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10mM HEPES, 2mM glutamine, 100U/L penicillin and 100µg/mL streptomycin and maintained at 37°C under 5% CO₂. In sterile 12 well plates (Corning, Sigma Aldrich, USA), 600,000 cells in 2 ml RPMI 1640 media were differentiated into macrophages by incubation with phorbol 12-myristate-13-acetate (PMA; Sigma Aldrich) to a final concentration of 5 ng/ml for 48 hours [131]. Subsequently, media supernatants were removed and wells were washed three times with sterile PBS to remove unattached cells and new RPMI 1640 media was added.

2.2.2 CD44, TLR2 and TLR4 expression in THP-1 macrophages

THP-1 macrophages were incubated with CD44-specific, TLR2-specific or TLR4-specific antibody (Abcam, USA) at a final concentration of 1µg per 600,000 cells for 1 hour at 4°C. Cells were subsequently pelleted and the cell pellet was washed three times with PBS. THP-1 cells were subsequently incubated with DyLight® 488 goat anti-mouse IgG (Abcam) at 1:500 dilution for 30 min at 4°C. Following cell pelleting and washing, 500 µL of 4% paraformaldehyde was added and cell-associated fluorescence was determined by flow cytometry using BD FACSVerser (BD Biosciences, USA).

2.2.3 Impact of TLR2 and TLR4 receptor activation on IL-1β and TNF-α production by THP-1 macrophages

THP-1 macrophages (600,000 cells per well) were treated with Pam3CSK4 (Pam; Invivogen, USA), a TLR2 ligand [132], at a concentration range between 0.1 and 10 ng/ml for 24 hours. Similarly, THP-1 macrophages (600,000 cells per well) were treated with lipopolysaccharide (LPS; Invivogen, USA), a TLR4 ligand, at a concentration range between 0.1 and 10 ng/ml for 24 hours. Media supernatants were collected and IL-1β and TNF-α concentrations were determined using commercially available ELISA kits (R&D systems, USA).

2.2.4 HA binding to recombinant human CD44 and TLR2 receptors

High-binding microtiter plates (Corning, Sigma Aldrich) were used to coat recombinant human CD44 Fc chimeric protein or recombinant human TLR2 (R&D systems) overnight at 4°C. Each well received 100µL of CD44 or TLR2 protein (1 ng/ml each) in PBS. Wells that were coated with CD44 or TLR2 were blocked with 2% bovine serum albumin (BSA; 300µL per well) for 2 hours at room temperature. Biotinylated HA (MW 1,500 kDa, Creative PEGWorks, USA) was added to the plate in serial dilutions and incubated for 1 hour at room temperature. Following washing with PBS+0.1% Tween 20, streptavidin-HRP (R&D systems) was added at 1:10,000 dilution and incubated for 1 hour at room temperature. Following washing with PBS+0.1% tween 20, the assay was developed using 1-step Turbo TMB ELISA reagent (Lifetechnologies, USA) and the absorbance was measured at 450 nm. Data is presented as percent maximal binding. Data represents the mean $\pm$ standard deviation (S.D.) of three independent experiments with duplicate wells per group.

2.2.5 Impact of HA and CD44-specific antibody treatments on TLR2 ligand induced nuclear factor kappa B (NFκB) nuclear translocation in THP-1 monocytes and macrophages

THP1-XBlue cells (Invivogen, USA) is a THP-1 monocyte cell line that stably expresses AP-1 and NFκB inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Upon activation of TLR2, SEAP is secreted and its activity can be monitored using an alkaline phosphatase substrate [133]. THP1-XBlue cells were cultured in RPMI 1640 media supplemented with 4.5 g/L glucose, 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL normocin and 2 mM L-glutamine.

THP1-XBlue cells (50,000 cells per well in HEK detection media) (100 µL per well) were treated with Pam (5 ng/ml) in the absence or presence of HA (MW >950 kDa; R&D systems) at a final

concentration of 250 and 500 µg/ml, an anti-CD44 antibody (2.5 µg/ml; Abcam) or an isotype control antibody (2.5 µg/ml; Abcam) for 24 hours followed by measuring the 630 nm absorbance.

THP-1 macrophages (600,000 cells per well) were treated with Pam (5 ng/ml) in the absence or presence of HA (100, 250 and 500 µg/ml) for 1 hour followed by cell harvesting and nuclear protein extraction using a commercially available kit (Thermo Fisher Scientific, USA). Protein content in the nuclear extract was quantified in all experimental groups and a total of 3 µg of total protein was used to quantify NFκB p65 subunit nuclear levels using a DNA binding ELISA assay (Abcam). In another set of experiments, THP-1 macrophages were pre-treated with okadaic acid (5nM; Tocris Biosciences, USA), a potent inhibitor of protein phosphatase 2A, for 2 hours followed by treatment with Pam (5ng/ml) in the absence or presence of HA (250 µg/ml) for 1 hour followed by nuclear NFκB p65 subunit quantification as described above. Data is presented as detectable nuclear p65 levels normalized to control. Data represents the mean ± S.D. of three independent experiments with duplicate wells per treatment.

2.2.6 Impact of HA and CD44-specific antibody treatments on TLR2 ligand induced IL-1β and TNF-α gene expression and production in THP-1 macrophages

THP-1 macrophages (600,000 cells per well) were treated with Pam (1 ng/ml) in the absence or presence of HA (100, 500 and 1,000 µg/ml) for 6 hours followed by RNA extraction using TRIzol reagent (Thermo Fisher Scientific) and RNA concentrations were determined using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, USA). cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). Quantitative PCR (qPCR) was performed on Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using TaqMan Fast Advanced Master Mix (Life Technologies). The cycle threshold (Ct) value of IL-1β (Hs00174097_m1; Thermo Fisher Scientific) was normalized to the Ct value

of GAPDH (Hs02758991_g1; Thermo Fisher Scientific) in the same sample, and the relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method [134]. Data is presented as fold target gene expression compared to untreated control.

In another set of experiments, THP-1 macrophages (600,000 cells per well) were treated with Pam (5ng/ml) in the absence or presence of HA (100, 500 and 1,000 μ g/ml), anti-CD44 antibody (2.5 μ g/ml) or isotype control antibody (2.5 μ g/ml) for 24 hours. IL-1 β and TNF- α media concentrations were determined using commercially available ELISA kits.

2.2.7 Impact of HA treatment on TLR2 ligand induced CD44 expression in THP-1 macrophages

THP-1 macrophages (600,000 cells per well) were treated with Pam (5 ng/ml) in the absence or presence of HA (100, 500 and 1,000 μ g/ml) for 6 hours followed by RNA isolation, cDNA synthesis and qPCR as described above. The C_t value of CD44 (Hs01075864_m1; Thermo Fisher Scientific) was normalized to the C_t value of GAPDH in the same sample, and the relative expression was determined as described above. Data is presented as fold CD44 expression compared to untreated control.

In another set of experiments, THP-1 macrophages were treated as described above for 24 hours followed by cell harvest. THP-1 macrophages were incubated with anti-CD44 antibody (1 μ g per 600,000 cells) for 1 hour at 4°C. Cells were subsequently pelleted and the cell pellet was washed three times with PBS. THP-1 cells were subsequently incubated with DyLight® 488 goat anti-mouse IgG at 1:500 dilution for 30 min at 4°C. Following cell pelleting and washing, cell-associated fluorescence was determined as described above.

2.2.8 CD44 receptor knockdown and associated TLR2 ligand induced IL-1 β and TNF- α production in THP-1 macrophages and role of HA

THP-1 macrophages (600,000 cells per well) were treated with a pre-validated CD44 small interfering RNA (siRNA) (Thermo Fisher Scientific) (25 pmoles per well) or a non-targeted negative control (NC) siRNA (25pmoles) (Thermo Fisher Scientific) for 48 hours. Transfection was performed using Lipofectamine RNAiMax (Thermo Fisher Scientific) per manufacturer's recommendations. To confirm CD44 knockdown, CD44 gene expression was determined in CD44 siRNA and NC siRNA-treated THP-1 macrophages as described above and compared to CD44 expression in untreated control macrophages. Additionally, CD44 and TLR2 protein levels in CD44 siRNA, NC siRNA-treated and untreated control THP-1 macrophages were determined using flow cytometry as described above.

NF κ B p65 subunit nuclear levels in CD44 siRNA, NC siRNA-treated and untreated control THP-1 macrophages (600,000 cells per well) following treatment with Pam (5ng/ml) for 1 hour were determined as described above. CD44 siRNA, NC siRNA-treated and untreated control THP-1 macrophages (600,000 cells per well) were treated with Pam (5ng/ml) for 24 hours. In another set of experiments, Pam treatment was performed in the absence or presence of HA (500 μ g/mL). IL-1 β and TNF- α media concentrations were determined as described above.

2.2.9 TLR2 ligand induced TNF- α production in murine bone marrow derived macrophages from *CD44*^{+/+} and *CD44*^{-/-} mice and effect of HA treatment on primary murine macrophages

CD44^{-/-} (JAX stock # 005085) and *CD44*^{+/+} (JAX stock # 00664) pathogen-free male mice (n=8 in each group) were acquired from the Jackson Laboratory (Maine, USA) [135]. Animals (10-14 weeks old) were euthanized and isolation and culture of bone marrow derived macrophages (BMDMs) was performed as previously described [136]. Both femurs and tibia bones were carefully dissected and bone marrows were flushed using a sterile 25G needle filled with

DMEM/F12 medium (Fisher Scientific) supplemented with 10ng/ml macrophage colony-stimulating factor (M-CSF; R&D systems). Cells were subsequently cultured for 7 days with media change on day 3. On day 7, BMDMs were gently scraped and dislodged using Corning Cellstripper Solution (VWR, USA) for 5 min at 37°C. An equal volume of macrophage complete medium was added to the cells and the cells were centrifuged for 10 min at 400 x g and 4°C, and the supernatant was discarded. Subsequently, BMDMs were plated to perform TLR2 stimulation studies or to characterize surface markers Cd11b and F4/80 expression using flow cytometry.

BMDMs (400,000 cells) were treated with FITC-labeled anti-CD11b antibody (abcam) (1:500 dilution), PE-Texas Red-labeled anti- F4/80 antibody (ThermoFisher Scientific) (1:500 dilution) or FITC-labeled Rat IgG2b, kappa monoclonal - Isotype control (Abcam) for 1 hr at 37°C. Cells were subsequently pelleted and the cell pellet was washed three times with PBS. Following cell pelleting and washing, 500 µL of 4% paraformaldehyde were added and cell-associated fluorescence was determined by flow cytometry.

BMDMs from both genotypes were plated overnight in 12 well plates (200,000 cells per well). Cells were treated with Pam (1 ng/ml or 5 ng/ml) for 6 hours at 37°C and TNF- α media concentrations were determined using a commercially available ELISA (R&D Systems). In another set of experiments, *CD44*^{+/+} BMDMs were treated with Pam (5 ng/ml) in the absence or presence of HA (100, 250 and 500 µg/ml) for 6 hours at 37°C and TNF- α media concentrations were determined as above. Data is presented as the mean TNF- α concentrations $\pm$ S.D. of 4 independent experiments, with triplicate wells per treatment. All animal tissue harvests were approved by the IACUC committee at Chapman University.

2.2.10 Activation of TLR2 and TLR4 receptors by synovial fluids from patients with OA and the role of CD44 in regulating macrophage activation in response to OA synovial fluids

Synovial fluid aspirates (SF) were collected from patients with OA (n=12) (Articular Engineering, USA) following knee replacement surgery or from donors within 24 hours of death, collected with partner site IRB approval with informed written consent from the donor or nearest relative. A total of 6 patients were female. The median age (interquartile range) of the group was 71 (60 to 82). Normal SF specimens (n=4) were obtained from subjects with no clinical history of joint disease or arthritis, and were provided by Dr. Martin Lotz from the Scripps Research Institute, USA. Screening of OA SF specimens for activation of TLR2 and TLR4 receptors was performed by incubation with TLR2-HEK and TLR4-HEK cells (25,000 cells per well in HEK Blue detection media) (Invivogen). The volume of SF aspirates was 7.5 μ L per well, corresponding to 3.75% dilution. The final volume in each well was 200 μ L. Activation of TLR2 or TLR4 in these cells results in nuclear translocation of NF κ B and expression of SEAP, whose activity can be detected in the culture media. Cells were incubated with the SF samples for 48 hours at 37°C. The 630 nm absorbance was measured and normalized to the 630 nm absorbance values of untreated control cells.

THP1-XBlue monocytes (25,000 cells per well) were maintained in serum-free RPMI 1640 for 48 hours followed by differentiation of the THP-1 monocytes into macrophages as described above. SF aspirates with detectable TLR2 and TLR 4 activity were incubated with THP1-XBlue macrophages for 48 hours in HEK Blue detection media in the absence or presence a CD44-specific antibody (2.5 μ g/ml), TLR2-specific antibody (Abcam; 2.5 μ g/ml) or isotype control (2.5 μ g/ml). Normal SF specimens were used as controls. The 630 nm absorbance was subsequently measured. Data is presented as the mean 630 nm absorbance value of each SF specimen, based on two independent experiments with duplicate wells per experiment.

2.2.11 Statistical Analyses

Unless otherwise specified, data is presented as the mean $\pm$ S.D. of 4 independent experiments with at least duplicate wells per group. Continuous variables were tested for normality and equal variances. Variables that satisfied both assumptions were tested for statistical significance using Student's *t*-test for two group comparisons. Multiple group comparisons were performed using analysis of variance with Tukey's post-hoc test. Variables that did not satisfy the normality assumption were tested using Mann-Whitney U test or ANOVA on the ranks. The level of statistical significance was *a priori* set at $\alpha=0.05$.

2.3 Results

2.3.1 CD44 and TLR receptor expression in human THP-1 macrophages and proinflammatory cytokine production

A representative flow cytometry histogram depicting cell-associated fluorescence of THP-1 macrophages following probing with CD44, TLR2 or TLR4 antibodies is shown in figure 2.1A. Compared to unstained cells, cell associated fluorescence for CD44 and TLR2 exhibited a right shift indicating expression of CD44 and TLR2 by THP-1 macrophages. On the contrary, cell associated fluorescence for TLR4 was not qualitatively different from unstained control cells. TLR2 receptor activation resulted in a concentration-dependent elevation in media IL-1 β (fig. 2.1B) and TNF- α (fig. 2.1C) concentrations. Similarly, TLR4 receptor activation resulted in detectable IL-1 β and TNF- α levels. Across all ligand treatments, TLR2 activation resulted in a significantly higher ($p<0.001$) IL-1 β and TNF- α production compared to TLR4 activation.

2.3.2 HA and CD44-specific antibody treatments reduced TLR2 ligand induced NFκB nuclear translocation in THP-1 monocytes and macrophages

HA binding to immobilized TLR2 and CD44 receptors is shown in figure 2.2A. HA exhibited concentration-dependent binding to CD44 with no detectable binding to TLR2. Pam treatment resulted in NFκB nuclear translocation in THP-1 monocytes compared to untreated cells ($p<0.001$) (fig. 2.2B). HA (250 and 500 µg/mL) treatment reduced NFκB nuclear translocation in THP-1 monocytes ($p<0.01$; $p<0.001$) following TLR2 receptor activation. Similarly, Anti-CD44 antibody treatment reduced NFκB nuclear translocation in THP-1 monocytes ($p<0.001$). In contrast, isotype control antibody treatment did not alter NFκB nuclear translocation. There was no difference in the magnitude of reduction of NFκB nuclear translocation in THP-1 monocytes with HA (500 µg/mL) or a CD44 antibody treatments.

TLR2 receptor activation resulted in NFκB p65 subunit nuclear translocation in THP-1 macrophages compared to untreated cells ($p<0.001$) (fig. 2.2C). HA (250 and 500 µg/mL) treatments reduced p65 subunit translocation ($p<0.01$) in THP-1 macrophages following TLR2 receptor activation. Okadaic acid treatment did not alter NFκB p65 subunit translocation following TLR2 receptor activation (fig. 2.2D). NFκB p65 subunit nuclear levels were significantly higher in the Pam + HA + Okadaic acid group compared to Pam + HA group ($p<0.01$).

2.3.3 HA and CD44-specific antibody treatments reduced TLR2 ligand stimulated proinflammatory cytokine expression and production in THP-1 macrophages

TLR2 activation induced IL-1β gene expression and production in THP-1 macrophages ($p<0.001$) (fig. 2.3A and fig. 2.3C). HA (500 and 1,000 µg/mL) treatment reduced IL-1β gene expression in THP-1 macrophages following TLR2 activation ($p<0.05$; $p<0.01$) (fig. 2.3A). Correspondingly, HA (500 and 1,000 µg/mL) treatment reduced IL-1β production by THP-1 macrophages ($p<0.001$) (fig. 2.3C). TLR2 activation induced TNF-α gene expression and production in THP-1

macrophages ($p < 0.001$) (fig. 2.3B and fig. 2.3D). HA (500 and 1,000 $\mu\text{g/mL}$) treatment reduced TNF- α gene expression in THP-1 macrophages following TLR2 activation ($p < 0.01$) (fig. 2.3B). Similarly, HA (500 and 1,000 $\mu\text{g/mL}$) treatment reduced TNF- α production by THP-1 macrophages ($p < 0.001$) (fig. 2.3D). CD44 antibody treatment reduced IL-1 β and TNF- α media supernatant concentrations following TLR2 activation ($p < 0.001$) (Fig. 2.3E and fig. 2.3F). In contrast, IL-1 β and TNF- α media concentrations were not significantly different between Pam alone and Pam + isotype control (IC) antibody groups.

2.3.4 HA reduced CD44 expression following TLR2 receptor activation in THP-1 macrophages

TLR2 activation induced CD44 gene expression in THP-1 macrophages ($p < 0.001$) (fig. 2.4A). HA (100, 500 and 1,000 $\mu\text{g/mL}$) treatment reduced CD44 expression in THP-1 macrophages following TLR2 activation ($p < 0.01$). A dose-response for HA treatment was observed as CD44 expression in the Pam + HA (1,000 $\mu\text{g/mL}$) group was significantly lower than CD44 gene expression in the Pam + HA (500 $\mu\text{g/mL}$) and Pam + HA (100 $\mu\text{g/mL}$) ($p < 0.01$; $p < 0.001$). A representative flow cytometry histogram depicting cell-associated fluorescence of THP-1 macrophages following probing with CD44 antibody is presented in figure 2.4B. TLR2 activation resulted in a right shift of the cell population indicating increased CD44 protein levels on macrophages. HA treatments showed a qualitative reduction in mean cell associated fluorescence, indicating a reduction in CD44 receptor levels on macrophages. Semi-quantitative analysis of mean cell-associated fluorescence is shown in figure 2.4C. Mean fluorescence in the Pam alone group was significantly higher compared to untreated control group ($p < 0.001$). Mean cell-associated fluorescence in the Pam + HA (1,000 $\mu\text{g/mL}$) and Pam + HA (500 $\mu\text{g/mL}$) were significantly lower than in the Pam group ($p < 0.001$). Similarly, mean cell-associated fluorescence

in the Pam + HA (1,000 µg/mL) and Pam + HA (500 µg/mL) were significantly lower than in the Pam + HA (100 µg/mL) group ($p < 0.01$).

2.3.5 Role of CD44 in regulating downstream responses of TLR2 activation in THP-1 macrophages

The impact of CD44 silencing on CD44 gene expression and protein is shown in figures 2.5A and 2.5B. CD44 silencing was achieved with approximately 85% reduction in CD44 gene expression and 60% reduction in CD44 protein. CD44 knockdown did not alter TLR2 expression in THP-1 macrophages (fig. 2.5C). CD44 knockdown resulted in a significant reduction in NFκB p65 subunit nuclear levels following TLR2 activation compared to NC siRNA-treated or control THP-1 macrophages ($p < 0.001$) (fig. 2.5D). CD44 knockdown resulted in a significant reduction in IL-1β and TNF-α production following TLR2 activation compared to NC siRNA-treated or control THP-1 macrophages ($p < 0.001$) (fig. 2.5E and 2.5F). There was no significant difference in Pam-stimulated IL-1β or TNF-α media concentrations between NC siRNA-treated and control THP-1 macrophages. HA (500 µg/mL) treatment reduced IL-1β and TNF-α media concentrations following TLR2 receptor activation in NC siRNA treated and control THP-1 macrophages ($p < 0.001$) (fig. 2.6A and 2.6B). In contrast, HA (500 µg/mL) treatment did not significantly alter IL-1β and TNF-α media concentrations following TLR2 receptor activation in CD44 siRNA-treated THP-1 macrophages.

2.3.6 CD44 modulated TLR2 ligand-induced TNF-α production by murine BMDMs and the HA treatment suppressed TLR2 ligand activation of primary murine BMDMs.

TNF-α production by murine BMDMs from *CD44*^{-/-} and *CD44*^{+/+} mice in response to TLR2 receptor activation and the impact of HA treatment is shown in figure 2.7. A representative flow cytometry scatterplot showing CD11b and F4/80 probing of murine BMDMs is presented in figure 2.7A. Murine BMDMs exhibited strong positive staining for both CD11b and F4/80 epitopes,

with typically 90% or more of the cell population positive for both surface markers. Using two-way ANOVA, we identified a significant interaction between CD44 genotype and Pam concentrations ($p=0.0035$). TNF- α media concentrations in the Pam (1ng/ml and 5 ng/ml) treated $CD44^{+/+}$ BMDMs were significantly higher compared to Pam-treated $CD44^{-/-}$ BMDMs (fig. 2.7B) ($p<0.001$; $p<0.01$). The mean TNF- α media concentration in the Pam (1ng/ml) treated $CD44^{-/-}$ BMDMs group was approximately 67% lower than corresponding mean TNF- α media concentrations in the Pam-treated $CD44^{+/+}$ BMDMs. Similarly, the mean TNF- α media concentration in the Pam (5ng/ml) treated $CD44^{-/-}$ BMDMs group was approximately 62% lower than the corresponding mean TNF- α media concentration in the Pam-treated $CD44^{+/+}$ BMDMs. In $CD44^{+/+}$ BMDMs, TNF- α concentrations in the Pam (1 and 5ng/ml) groups were significantly higher than control group ($p<0.001$). Additionally, TNF- α concentration in the Pam (5ng/ml) group was significantly higher than the Pam (1ng/ml) group ($p<0.001$). In $CD44^{-/-}$ BMDMs, TNF- α concentrations in the Pam (1ng/ml) group was not significantly different from control $CD44^{-/-}$ BMDMs ($p=0.1207$). Furthermore, TNF- α concentration in the Pam (5ng/ml) group was significantly higher than the Pam (1ng/ml) group ($p<0.001$).

The impact of HA treatment on TLR2 ligand induced TNF- α production in $CD44^{+/+}$ murine BMDMs is shown in figure 2.7C. TNF- α media concentrations in the Pam + HA (100 μ g/ml) group were not significantly different from TNF- α media concentrations in the Pam alone group. TNF- α media concentrations in the Pam + HA (250 μ g/ml) group were significantly lower than TNF- α media concentrations in the Pam alone and Pam + HA (100 μ g/ml) groups ($p<0.001$). Similarly, TNF- α media concentrations in the Pam + HA (250 μ g/ml) group were significantly lower than TNF- α media concentrations in the Pam alone and Pam + HA (100 μ g/ml) groups

($p < 0.001$). HA (500 $\mu\text{g/ml}$) alone treatment did not alter TNF- α production compared to untreated control cells.

2.3.7 OA SF specimens activated TLR2 and TLR4 receptors and a CD44-specific antibody reduced activation of THP-1 macrophages by OA SF specimens.

Activation of TLR2 and TLR4 receptors by OA SF specimens is shown in figure 2.8A. A total of 8 samples demonstrated significant activation of TLR2 and TLR4 receptors ($p < 0.001$). In contrast, 3 specimens did not exhibit activation of TLR2 or TLR4 and one sample activated TLR2 receptor but not the TLR4 receptor. OA SF specimens that activated TLR2 and TLR4 receptors also significantly stimulated NF κ B nuclear translocation in THP-1 macrophages compared to normal SF specimens ($p < 0.001$) (fig. 2.8B). TLR2 neutralizing antibody treatment significantly reduced OA SF activation of THP-1 human macrophages ($p < 0.01$). Similarly, CD44 antibody treatment significantly reduced OA SF activation of THP-1 human macrophages ($p < 0.01$).

2.4 Discussion

The TLR family comprises ten functional receptor subtypes with TLR 1-7 and 9 being detected in the synovial tissues of patients with OA [107, 118, 137, 138]. A role for TLRs, specifically TLR2 and TLR4, in the pathogenesis of OA has been suggested [139, 140]. Progressive OA was associated with expression of TLR2 in cartilage and chondrocytes derived from a TLR2/TLR4 double knockout mouse showed attenuated matrix metalloproteinase-13 (MMP-13) expression in response to TLR2 stimulation [139, 140]. Furthermore, evidence of macrophage activation in the synovial lining was recently reported and the extent of synovial macrophage activation was shown to be associated with OA severity and joint pain [141]. In this work, we have examined macrophage activation in response to TLR2 and TLR4 activation. TLR2 and TLR4 ligands induced gene expression and production of IL-1 β and TNF- α in a concentration-dependent

manner. TLR2 activation produced higher levels of IL-1 β and TNF- α compared to TLR4 across all ligand concentrations utilized. This may be due to the relative level of TLR2 and TLR4 expression on the surface of the macrophages. Using flow cytometry, we have detected increased TLR2 expression compared to TLR4. This finding is in line with other work that demonstrated enhanced TLR2 expression on THP-1 macrophages [142]. TLR2 activation resulted in NF κ B nuclear translocation in a monocyte NF κ B reporter assay. In macrophages, TLR2 ligands induced the nuclear translocation of NF κ B p65 subunit. In addition to the induction of proinflammatory cytokines, TLR2 activation significantly increased CD44 gene expression and increased CD44 protein levels.

CD44 is a transmembrane receptor, with various isoforms generated by extensive alternative splicing and post-translational modifications [143]. In addition to its established role in mediating cell adhesion and migration, CD44 receptor has a role in regulating cell signaling pathways, by facilitating signaling protein recruitment and assembly [144]. In the context of innate immunity, CD44 was found to regulate Fc γ and complement receptor 3-dependent macrophage phagocytosis [144]. Additionally, CD44 may play a role in the negative regulation of TLR receptor activation [145, 146]. In this present study, a CD44 antibody treatment reduced NF κ B nuclear translocation and downstream IL-1 β and TNF- α gene expression and production. Additionally, reducing CD44 expression in human macrophages resulted in a significant attenuation of the latter's response to TLR2 receptor activation and downstream proinflammatory response. This attenuation is not related to the TLR receptor density on the surface of macrophages, as CD44 receptor knockdown did not modify TLR2 receptor density on THP-1 macrophages. The regulatory role of CD44 is further highlighted by a strongly attenuated proinflammatory response following TLR2 receptor

stimulation of primary macrophages derived from CD44 knockout mice compared to macrophages derived from CD44 wildtype animals.

Protein phosphatase-2A (PP2A) is an abundant intracellular serine/threonine phosphatase with key roles in the regulation of many cellular functions including cellular proliferation and immune responses [147, 148]. Inhibition of PP2A by okadaic acid resulted in increased nuclear translocation of NF κ B and AP-1 and IL-1 β expression in THP-1 macrophages [147]. Additionally, CD44 engagement was shown to increase intracellular PP2A [149]. In our work, HA suppressed NF κ B p65 subunit nuclear translocation in response to TLR2 activation, and that effect was shown to be CD44 dependent as HA failed to demonstrate an anti-inflammatory effect in response to TLR2 activation in macrophages following CD44 knockdown. The suppressive effect of HA was confirmed in primary murine macrophages as HA treatment dose-dependently reduced TNF- α production subsequent to TLR2 receptor stimulation. Furthermore, the effect of HA was mediated by intracellular PP2A activity, as inhibition of PP2A activity reduced the inhibitory effect of HA on NF κ B activation. The observed anti-inflammatory activity of HA is not due to a direct interaction between HA and the TLR2 receptor as we did not observe any significant binding of HA to recombinant TLR2 receptor. The biological effect of HA in reducing TLR2 mediated proinflammatory response in macrophages is physiologically relevant. The concentration of HA in normal SF can vary between 2 and 4 mg/ml [150]. In OA SF aspirates, the concentration and molecular weight distribution of HA are significantly reduced, and this reduction was associated with TLR2 and TLR4 activation by OA SF aspirates [150, 151]. This association may argue for an endogenous homeostatic role for HA in inhibiting synovial macrophage activation in response to TLR receptor stimulation by cartilage matrix degradation products.

A majority of the aspirated SF samples from patients with advanced OA that we examined activated TLR2 and TLR4 receptors. This observation is in agreement with previous reports demonstrating activation of TLR2 and TLR4 receptors by OA SF, and augmentation of TLR-mediated responses in OA fibroblast-like synoviocytes by SF from patients with early stage OA [151-153]. Interestingly, SF specimens that activated TLR2 and TLR4 receptors induced NF κ B nuclear translocation in macrophages, while normal SF specimens treatments failed to activate macrophages. Macrophage TLR2 receptors appeared to mediate NF κ B nuclear activation as neutralization of TLR2 abrogated macrophage activation by these SF specimens. CD44 antibody treatment produced a similar effect to TLR2 neutralization, providing further support to the utility of targeting CD44 to suppress macrophage response to DAMPs present in OA SF.

We have demonstrated that CD44 receptor plays a significant role in suppressing TLR2-linked NF κ B nuclear translocation and resultant proinflammatory response in macrophages, in a mechanism that involves intracellular PP2A. Neutralization of the CD44 receptor by its ligand hyaluronan, that binds to and is internalized by different cell types including macrophages [127], or by a monoclonal antibody resulted in inhibition of NF κ B nuclear translocation. Absence of the CD44 receptor or its knockdown produced a similar effect to receptor neutralization by hyaluronan or an antibody. OA SF activated TLR2 and TLR4 receptors and correspondingly induced NF κ B nuclear translocation in a mechanism that involves TLR2. Otherwise, a CD44-specific antibody reduced macrophage activation by these SF specimens. In conclusion, CD44 is a potentially novel target that may act to limit synovial macrophage activation by cartilage matrix degradation products in the joint.

This study expands the role of CD44 in OA pathogenesis. CD44 can bind different types of ligands and in addition to HA, CD44 was shown to bind proteoglycan-4 (PRG4), a major component of

synovial fluids [154]. CD44 expression was shown to be associated with enhanced proliferation of synoviocytes from patients with OA, rheumatoid arthritis (RA) as well synoviocytes from *Prp4*^{-/-} mice. Targeting the CD44 receptor by HA or by the recombinant form of PRG4 reduced cytokine-induced OA and RA synoviocyte proliferation. The regulatory role of CD44 in controlling downstream effects of IL-1 β was further confirmed by the ability of PRG4 to inhibit I κ B phosphorylation, NF κ B nuclear translocation and expression of cartilage degrading enzymes by OA synoviocytes in a CD44-dependent mechanism [155]. Our findings are limited by the low number of SF aspirates that we have investigated. Furthermore, we did not ascertain the molecular identity of the DAMPs in the SF aspirates that activated TLR receptors and induced NF κ B nuclear translocation in THP-1 macrophages.

2.5 Acknowledgements

The authors would like to thank Dr. Martin Lotz for providing normal synovial fluids.

2.6 Figures

Fig. 2.1 Toll-like receptors 2 and 4 (TLR2 and TLR4) and CD44 receptor expression in THP-1 macrophages and impact of TLR receptor stimulation on proinflammatory cytokine production. Data represents the mean $\pm$ standard deviation of 4 independent experiments. $*p < 0.001$

A) A representative flow cytometry histogram of human THP-1 macrophages demonstrating enhanced TLR2 and CD44 receptor expression, compared to TLR4.

B) Impact of Pam3CSK4 (Pam; TLR2 ligand) and Lipopolysaccharide (LPS; TLR4 ligand) treatments on interleukin-1 beta (IL-1 β) production in THP-1 macrophages.

C) Impact of Pam3CSK4 and LPS treatment on tumor necrosis factor alpha (TNF- α) production in THP-1 macrophages.

Fig. 2.2 Binding of hyaluronan (HA) to recombinant human CD44 and TLR2 receptors and impact of HA treatment on TLR2 receptor stimulated nuclear translocation of nuclear factor kappa B (NFκB). TLR2 receptor stimulation was performed using Pam3CSK4 (Pam; 5 ng/mL).

A) Concentration-dependent binding of HA to immobilized CD44 or TLR2 receptors using a microplate assay format. HA exhibited a concentration-dependent binding to CD44 receptor and no significant binding to TLR2 receptor.

B) Impact of HA treatment (250 and 500 μ g/mL), a CD44 antibody (CD44 Ab 2.5 μ g/mL) or an isotype control (IC; 2.5 μ g/mL) on TLR2 induced NFκB nuclear translocation in THP1 XBlue monocytes. HA and CD44 Ab treatments reduced TLR2 induced NFκB nuclear translocation.

Data represents the mean $\pm$ standard deviation of 4 independent experiments. * $p < 0.001$; ** $p < 0.01$.

C) Impact of HA treatment (100, 250 and 500 $\mu\text{g/mL}$) on TLR2 induced NF κ B p65 subunit nuclear translocation in THP-1 macrophages. HA (250 and 500 $\mu\text{g/mL}$) treatments reduced NF κ B nuclear translocation in THP-1 macrophages. Data represents the mean $\pm$ standard deviation of 4 independent experiments. * $p < 0.001$; ** $p < 0.01$.

D) Protein phosphatase 2A (PP2A) mediates HA's inhibition of NF κ B p65 subunit nuclear translocation in THP-1 macrophages. Okadaic acid (OKA; 5 nM), a potent inhibitor of PP2A, abolished the inhibitory effect of HA (250 $\mu\text{g/mL}$) on NF κ B p65 subunit translocation in THP-1 macrophages. Data represents the mean $\pm$ standard deviation of 4 independent experiments. * $p < 0.001$; ** $p < 0.01$.

Fig. 2.3 Impact of hyaluronan (HA) (100, 500 and 1,000 $\mu\text{g/mL}$), CD44 antibody (CD44 Ab; 2.5 $\mu\text{g/mL}$) or isotype control (IC; 2.5 $\mu\text{g/mL}$) treatments on TLR2 receptor induced interleukin-1 beta (IL-1 β) or tumor necrosis factor alpha (TNF α) gene expression and production in THP-

macrophages. Data represents the mean $\pm$ standard deviation of 4 independent experiments.
p*<0.001; *p*<0.01; ****p*<0.05.

- A)** HA (500 and 1,000 $\mu\text{g/mL}$) treatments reduced TLR2 induced IL-1 β gene expression.
- B)** HA (500 and 1,000 $\mu\text{g/mL}$) treatments reduced TLR2 induced TNF- α gene expression.
- C)** HA (500 and 1,000 $\mu\text{g/mL}$) treatments reduced TLR2 induced IL-1 β production.
- D)** HA (500 and 1,000 $\mu\text{g/mL}$) treatments reduced TLR2 induced TNF- α production.
- E)** CD44 Ab treatment reduced TLR2 induced IL-1 β production.
- F)** CD44 Ab treatment reduced TLR2 induced TNF- α production.

Fig. 2.4 Impact of toll-like receptor 2 (TLR2) receptor stimulation on CD44 gene expression and CD44 levels in THP-1 macrophages and the role of hyaluronan (HA). TLR2 receptor stimulation was performed using Pam3CSK4 (Pam; 5ng/mL) for 24 hours. Data represents the mean $\pm$ standard deviation of 4 independent experiments. * $p < 0.001$; ** $p < 0.01$.

A) HA (100, 500 and 1,000 $\mu\text{g/mL}$) treatments reduced TLR2 induced CD44 gene expression in THP-1 macrophages.

B) A representative flow cytometry histogram demonstrating increased CD44 protein levels in TLR2 stimulated THP-1 macrophages. HA (500 and 1,000 $\mu\text{g/mL}$) treatments reduced CD44 levels in TLR2 stimulated THP-1 macrophages.

C) Semi-quantitative analysis of mean fluorescence intensities of CD44 receptor following TLR2 stimulation in the absence or presence of HA (100, 500 or 1,000 $\mu\text{g/mL}$). TLR2 stimulation

increased CD44 protein in THP-1 macrophages and HA (500 and 1,000 $\mu\text{g/mL}$) treatments reduced CD44 protein.

Fig. 2.5 Impact of CD44 receptor knockdown on toll-like receptor 2 (TLR2) receptor stimulated proinflammatory cytokine production in THP-1 macrophages and nuclear factor kappa B (NF κ B) p65 subunit nuclear translocation. TLR2 receptor stimulation was performed using Pam3CSK4 (Pam; 5ng/mL). Data represents the mean $\pm$ standard deviation of 4 independent experiments. * $p < 0.001$. *** $p < 0.05$.

A) CD44 siRNA treatment resulted in reduced CD44 gene expression compared to negative control (NC) siRNA treatment or control.

B) A representative flow cytometry histogram demonstrating reduced CD44 protein levels in CD44 siRNA-treated THP-1 macrophages compared to control.

C) Semi-quantitative analysis of mean fluorescence intensities of CD44 or TLR2 receptors in THP-1 macrophages following CD44 knockdown. CD44 knockdown resulted in approximately 60% reduction in CD44 levels.

D) CD44 siRNA treatment reduced TLR2 stimulated NF κ B p65 subunit nuclear translocation.

E) CD44 siRNA treatment reduced TLR2 stimulated interleukin-1 beta (IL-1 β) production in THP-1 macrophages.

F) CD44 siRNA treatment reduced TLR2 stimulated tumor necrosis factor alpha (TNF- α) production in THP-1 macrophages.

Fig. 2.6 Impact of hyaluronan (HA) treatment on toll-like receptor 2 (TLR2) induced interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) production in CD44 siRNA-treated, negative control (NC) siRNA-treated and untreated control THP-1 macrophages. TLR2 stimulation was performed using Pam3CSK4 (Pam; 5ng/mL) for 24 hours. HA treatment was performed at 500 μ g/mL. Data represents the mean $\pm$ standard deviation of 4 independent experiments. * $p < 0.001$; n.s.: not significant.

A) HA treatment reduced TLR2 induced IL-1 β production in untreated control and NC siRNA-treated THP-1 macrophages. HA treatment did not alter IL-1 β levels in CD44 siRNA-treated macrophages.

B) HA treatment reduced TLR2 induced TNF- α production in untreated control and NC siRNA-treated THP-1 macrophages. HA treatment did not alter TNF- α levels in CD44 siRNA-treated macrophages.

Fig. 2.7 Impact of toll-like receptor 2 (TLR2) receptor stimulation on tumor necrosis factor alpha (TNF- α) production by *CD44*^{+/+} and *CD44*^{-/-} murine bone marrow derived macrophages (BMDMs) and dose-dependent effect of HA on *CD44*^{+/+} BMDMs. TLR2 stimulation was performed using Pam3CSK4 (Pam; 5ng/mL) for 6 hours. Data represents the mean $\pm$ standard deviation of 4 independent experiments. * p < 0.001; ** p < 0.01.

A) A representative flow cytometry histogram of murine BMDMs showing positive staining for CD11b and F4/80 surface markers.

B) TLR2 receptor activation dose-dependently increased TNF- α production by murine *CD44*^{+/+} BMDMs compared to *CD44*^{-/-} BMDMs.

C) HA (250 and 500 $\mu\text{g/mL}$) treatments reduced TLR2 induced TNF- α production by murine $CD44^{+/+}$ BMDMs.

Fig. 2.8 Activation of toll-like receptors 2 and (TLR2 and TLR4) by synovial fluid (SF) aspirates from patients with advanced osteoarthritis (OA) and role of CD44 in modulating THP-1 macrophages activation by OA SF. OA SF (3.75 μL per well) were incubated with TLR2-HEK or TLR4-HEK cells in HEK detection media for 48 hours followed by measuring the 630 nm absorbance. THP-1XBlue macrophages were incubated with OA SF in the absence or presence of a TLR2-specific, CD44-specific, or isotype control (IC) antibody (2.5 $\mu\text{g/mL}$) for 48 hours followed by measuring 630 nm absorbance. Normal SF specimens were used as controls. * $p < 0.001$; ** $p < 0.01$.

A) Activation of TLR2 and TLR4 receptors by OA SF (n=12). A total of 8 samples activated both TLR2 and TLR4 receptors, compared to untreated controls.

B) Activation of THP-1XBlue macrophages by OA SF (n=8) and the impact of CD44-specific or TLR2-specific antibody treatments. CD44 and TLR2 antibody treatments reduced OA SF induced macrophage activation.

3 CHAPTER 3

cAMP Attenuates TGF- β 's Profibrotic Responses in Osteoarthritic Synoviocytes: Involvement of Hyaluronan and PRG4

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ABSTRACT

Osteoarthritis (OA) is characterized by synovitis and synovial fibrosis. Synoviocytes are fibroblast-like resident cells of the synovium that are activated by TGF- β to proliferate, migrate and produce extracellular matrix. Synoviocytes secrete hyaluronan (HA) and proteoglycan-4 (PRG4). HA reduced synovial fibrosis *in vivo* and the *Prg4*^{-/-} mouse exhibits synovial hyperplasia. We investigated the antifibrotic effects of increased intracellular cAMP in TGF- β stimulated human OA synoviocytes. TGF- β 1 stimulated collagen I (COL1A1), α -SMA, TIMP-1, PLOD2 expression and procollagen I, α -SMA, HA and PRG4 production, migration and proliferation of OA synoviocytes were measured. Treatment of OA synoviocytes with forskolin (10 μ M) increased intracellular cAMP levels and reduced TGF- β 1 stimulated COL1A1, α -SMA and TIMP-1 expression, with no change in PLOD2 expression. Forskolin also reduced TGF- β 1 stimulated procollagen I and α -SMA content, as well as synoviocyte migration and proliferation. Forskolin (10 μ M) increased HA secretion, PRG4 expression and production. A cell permeable cAMP analog reduced COL1A1 and α -SMA expression and enhanced HA and PRG4 secretion by OA synoviocytes. HA and PRG4 reduced α -SMA expression and content and PRG4 reduced COL1A1 expression and procollagen I content in OA synoviocytes. *Prg4*^{-/-} synovium exhibited increased α -SMA, COL1A1 and TIMP-1 expression as compared to *Prg4*^{+/+} synovium. *Prg4*^{-/-} synoviocytes demonstrated strong α -SMA and collagen type I staining while these were undetected in *Prg4*^{+/+} synoviocytes, and was reduced with PRG4 treatment. We conclude that increasing intracellular cAMP levels in synoviocytes mitigates synovial fibrosis through enhanced production of HA and PRG4, possibly representing a novel approach for treatment of OA synovial fibrosis.

Keywords: cAMP, fibrosis, PRG4, HA, Osteoarthritis, synoviocytes.

3.1 INTRODUCTION

Hallmarks of osteoarthritis (OA) include cartilage degeneration, subchondral bone remodeling, and synovitis [112, 118, 156]. Major abnormalities in the OA synovium include synovial hyperplasia, inflammatory cell infiltration, angiogenesis and fibrosis [157-160]. Synovial fibrosis is a common feature in advanced OA that contributes to joint pain and stiffness [110, 161]. The TGF- β 1 family and its associated signaling pathways play an essential role in maintaining homeostasis in healthy joints [162]. However, TGF- β 1 switches to a pathologic role in OA joints that drives synovial fibrosis [163]. TGF- β 1 upregulates the expression of synovial collagen type 1, tissue inhibitor of metalloproteinase 1 (TIMP-1), and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) [163, 164]. The net result is an increase in collagen I formation, an increase in collagen crosslinks and a reduction in collagen turnover [163, 164].

The normal synovium contains two types of intimal cells: type A macrophages and type B fibroblasts, or synoviocytes [115]. In synoviocytes, TGF- β 1 induces fibrotic changes characterized by cell proliferation and collagen type I accumulation [165]. Additionally, TGF- β 1 promotes the differentiation of OA synoviocytes into a myofibroblast-like phenotype, characterized by the expression of alpha smooth muscle actin (α -SMA) [166]. Synoviocytes produce hyaluronan (HA), a glycosaminoglycan synthesized by membrane-bound hyaluronan synthase (HAS) with three isoforms identified to date (HAS1, HAS2 and HAS3) [167]. Synoviocytes also produce proteoglycan-4 (PRG4), a heavily glycosylated mucinous glycoprotein [168, 169]. HA and PRG4 play important roles in joint lubrication [170]. HA and PRG4 may also play a role in joint fibrosis as HA treatment reduced synovial fibrosis *in vivo* and findings in

synovial tissues from *Prp4*^{-/-} mice include increased synovial thickening and a proliferative capacity for *Prp4*^{-/-} synoviocytes under basal and cytokine stimulated conditions [81, 92, 154].

Cyclic adenosine monophosphate (cAMP) is a pleiotropic intracellular second messenger generated by adenylyl cyclase (AC) enzymes in response to G-protein coupled receptor (GPCR) activation [62]. The antifibrotic effect of cAMP has been described in fibroblasts from multiple origins and include inhibition of fibroblast proliferation, reduction in fibroblast migration and reduced synthesis of extracellular matrix components [171-174]. The role of cAMP in regulating TGF- β 1's fibrotic response in synoviocytes is unknown. Our aim was to study the impact of forskolin, an AC activator, on the expression and production of α -SMA, collagen type I, and expression of *TIMP-1*, *PLOD2*, *HAS* isoforms, *PRG4* and production of HA and PRG4 in a model of TGF- β 1 stimulated OA synoviocytes. Given the potential involvement of HA and PRG4 in synovial fibrosis, we also studied the antifibrotic effect of HA and PRG4 in human and murine synoviocytes. We hypothesized that increasing intracellular cAMP exerts an antifibrotic effect in OA synoviocytes and promotes HA and PRG4 production.

3.2 MATERIALS AND METHODS

3.2.1 Ethical approvals:

Animal breeding and tissue harvest was approved by the IACUC committee at Rhode Island Hospital.

3.2.2 Patient characteristics and experimental approach

OA synoviocytes (500,000 cells per vial; Cell Applications, USA) were isolated from synovial tissues from de-identified OA patients undergoing knee replacement (n=10; median age = 63; range: 54 to 69). Six patients were female. Five patients were Caucasian, and the other five were

unspecified. Synoviocytes were received in their second passage. OA Synoviocytes were cultured in 75 cm² culture flasks in DMEM media supplemented with 10% FBS and were used between the third and sixth passages to avoid alterations in patterns of gene expression [175, 176]. Experimental data are represented as the mean $\pm$ S.D. of 3-6 biological replicates.

We initially studied cAMP generation in OA synoviocytes using a 0.01 to 10 μ M forskolin concentration range. The 10 μ M was selected based on previous studies [177, 178]. Following confirmation of cAMP accumulation by forskolin, we determined its effect on α -SMA, collagen type I, TIMP-1 and PLOD2 expression in TGF- β 1 stimulated OA synoviocytes. The antifibrotic effect of intracellular cAMP accumulation in OA synoviocytes was further confirmed using a cell permeable cAMP analog, 8-bromo cAMP. To further appreciate the antifibrotic effect of forskolin, we evaluated the impact of cAMP generation on HAS isoform expression and HA production, as well as PRG4 expression and production. Based on forskolin's observed effect on HA and PRG4, we studied the antifibrotic effect of HA and PRG4 in human OA synoviocytes, murine *Prg4*^{-/-} synoviocytes as well as fibrosis markers in the *Prg4*^{-/-} synovium. Finally, we studied the efficacy of forskolin in mitigating TGF- β 1 stimulated OA synoviocyte migration and proliferation.

3.2.3 cAMP generation in OA synoviocytes by forskolin

cAMP levels were measured in OA synoviocytes using the cADDis assay (Montana Molecular, USA). The assay utilizes a fluorescent cAMP sensor that measures changes in intracellular cAMP levels. An increase in intracellular cAMP levels results in a reduction in green fluorescence. OA synoviocytes (10,000 cells per well) were cultured overnight in sterile 96-well clear-bottom black plates in the presence of a recombinant mammalianized baclovirus expressing the cAMP sensor

according to manufacturer's recommendations. Subsequently, media supernatants were replaced with Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, USA) (200µL per well). Forskolin (Sigma-Aldrich, USA) at a final concentration of 0.01, 0.1, 1, and 10µM, vehicle and a positive control were added and fluorescence intensity using 494/522 nm wavelengths was measured every 30 seconds over 30 minutes. Data is presented as the ratio of fluorescence intensity reduction at each time point to fluorescence intensity at baseline.

3.2.4 Gene expression studies

OA synoviocytes (300,000 cells per well) were treated with TGF-β1 (1ng/mL; R&D systems, USA) in serum-free DMEM ± forskolin (0.1, 1, or 10µM), 8-bromo cAMP (Sigma Aldrich) (100 and 500µM), human synoviocyte PRG4 (apparent MW 280 kDa as a monomer; 100µg/mL) [179] and/or high molecular weight HA (MW >950 kDa; R&D Systems) (100 µg/mL) for 24 hours followed by RNA extraction, cDNA synthesis and qPCR as previously described [155]. The cycle threshold (Ct) value of target genes were normalized to the Ct value of *GAPDH* in the same sample, and the relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method [134]. Target genes included α-SMA (*ACTA2*), collagen type I (*COL1A1*), *TIMP-1*, *PLOD2*, *HAS1*, *HAS2*, *HAS3*, and *PRG4* (primers and probes were obtained from Thermo Fisher Scientific). Data are presented as fold expression of target genes in the different experimental groups compared to untreated controls.

3.2.5 α-SMA and procollagen type I quantitation

OA synoviocytes were seeded in cell culture dishes (20.8 cm²) at 1.0×10^6 cells per dish until confluence. OA synoviocytes in serum-free DMEM media (5 ml per dish) were treated with TGF-β1 (1ng/mL) ± forskolin (10µM), PRG4 (100µg/mL) or HA (100µg/mL) for 24 hours. Cell protein extraction was performed using M-PER reagent supplemented with protease and phosphatase

cocktail inhibitor (Thermo Fisher Scientific) and quantified using micro BCA assay (Thermo Fisher Scientific).

Gel electrophoresis was performed using 10% PAGE gels (Bio-Rad) and 10 μ g protein per well. Following transfer, membranes were blocked with 5% non-fat dry milk for 2 hours at room temperature. Membranes were probed with anti- α -SMA (1:1,000 dilution) and anti-GAPDH (1:5,000 dilution) (Abcam) overnight in tris-buffered saline tween 20 (TBS-T). Following washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:5,000 dilution) antibody for 1 hour at room temperature (Abcam). Protein bands were developed using Lumigen ECL Ultra reagent (Lumigen, USA) and visualized using Bio-Rad ChemiDoc XRS+ system (Bio-Rad). Bands of interest were selected and quantified using Image J software. The ratio of α -SMA band intensities to corresponding GAPDH band intensities of the different experimental groups were calculated and normalized to controls.

Procollagen I content in OA synoviocytes was determined using an ELISA (Abcam and R&D Systems). A total of 5 μ g protein in 100 μ L buffer was used in each experimental group and procollagen I concentrations (pg/mL) were divided by 50 and expressed as procollagen I protein per μ g protein.

3.2.6 Immunocyto staining of α -SMA in OA synoviocytes

OA synoviocytes (200,000 cells per well) were cultured on collagen type I-coated 12 mm glass coverslips for 48 hours in DMEM supplemented with 10% FBS. Subsequently, cells were treated with TGF- β 1 (1 ng/ml) $\pm$ forskolin (10 μ M) for 48 hours in serum-free DMEM. Synoviocytes were fixed in 10% neutral buffered formalin for 10 min followed by washing twice with PBS. Cells

were permeabilized for 5 min using 0.01% Triton X100 in PBS and blocked using 2%BSA for 1 hour at room temperature. Probing was performed using FITC-conjugated anti α -SMA antibody (1:100; Abcam) and Alexa Fluor 594 conjugated anti-alpha tubulin antibody (1:500; Abcam) overnight at 4°C. Following washing with PBS, cells were mounted with DAPI mounting medium (Abcam) for 1 hour and viewed under a confocal microscope.

3.2.7 HA, PRG4 and HAS1 quantitation

HA concentrations: OA synoviocytes (300,000 cells per well) in serum-free DMEM were treated with TGF- β 1 (1ng/mL) $\pm$ forskolin (10 μ M) for 24 hours. Media supernatants were collected and assayed for HA using a quantitative assay kit (R&D systems). In a separate set of experiments, OA synoviocytes (300,000 cells per well) in serum-free DMEM were treated with TGF- β 1 (1ng/mL) $\pm$ 8-bromo cAMP (100 and 500 μ M) for 24 hours. HA concentrations were determined as described above.

PRG4 concentrations: OA synoviocytes (20,000 cells per well) were seeded in sterile 96 well plates for 48 hours followed by treatment with TGF- β 1 (1ng/mL) $\pm$ forskolin (10 μ M) for 48 hours. PRG4 concentrations, normalized to cell density, were determined in media supernatants as previously described [155]. In a separate set of experiments, OA synoviocytes (300,000 cells per well) in serum-free DMEM were treated with TGF- β 1 (1ng/mL) $\pm$ 8-bromo cAMP (100 and 500 μ M) for 24 hours. PRG4 concentrations were determined as described above.

HAS1 content: OA synoviocytes were seeded and treated as described for α -SMA and procollagen type I. HAS1 content in protein isolates were determined using an ELISA (MyBioSource, USA). A total of 1 μ g total protein in 100 μ L volume was added to the wells of the ELISA plate. HAS1

concentrations (ng/mL) were divided by 10 and expressed as HAS1 content (ng) per μ g total protein.

3.2.8 *HAS1* knockdown and its impact on forskolin-induced HA secretion in OA synoviocytes

OA synoviocytes (300,000 cells per well) in Opti-MEM reduced serum medium (Thermo Fisher Scientific) were treated with a *HAS1* small interfering RNA (siRNA) (Thermo Fisher Scientific) (25 pmoles per well) or a non-targeted negative control (NC) siRNA (25 pmoles per well) (Thermo Fisher Scientific) for 48 hours. Transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) per manufacturer's recommendations. To confirm *HAS1* knockdown, *HAS1* expression was determined as described above. In a separate set of experiments, *HAS1* knockdown in OA synoviocytes was performed followed by media change to serum-free DMEM and stimulation with TGF- β 1 (1ng/mL) $\pm$ forskolin (10 μ M) for 24 hours. Subsequently, HA concentrations were determined in media supernatants as described above.

3.2.9 Gene expression studies in *Prg4*^{+/+} and *Prg4*^{-/-} synovial tissues and immunocyto staining of murine synoviocytes

The phenotype of the *Prg4*^{-/-} mouse has been previously reported [180], and is characterized by cartilage degeneration and a hyperplastic synovium contributing to joint failure [180]. The *Prg4*^{-/-} and *Prg4*^{+/+} mouse colonies are maintained by Dr. Jay at Rhode Island Hospital. *Prg4*^{-/-} mouse is also commercially available (stock #025737; The Jackson Laboratory, Maine, USA). Synovial tissues were isolated from male *Prg4*^{-/-} and *Prg4*^{+/+} mice (8-10 weeks old). The skin and surrounding tissues of the knee joints were removed. The joint capsule was cut open along both sides of the patella under a stereo microscope and the synovium from the lateral and medial sides was carefully isolated. A total of 15 *Prg4*^{+/+} and 15 *Prg4*^{-/-} male mice were used in this study. Synovial tissues were harvested from every mouse and tissues from 3 mice were randomly pooled

into one sample, generating 5 pooled samples in each genotype. RNA isolation, cDNA synthesis and qPCR were performed as previously described. Genes of interest included *ACTA2*, *COL1A1*, *TIMP-1* and *PLOD2* with *GAPDH* as an internal reference gene (Thermo Fisher Scientific).

Prg4^{+/+} and *Prg4*^{-/-} synoviocytes were isolated as previously described [86]. Synovial tissues from male *Prg4*^{+/+} and *Prg4*^{-/-} mice (15 animals per genotype) were used to isolate the synoviocytes. Synoviocytes were plated onto sterile chamber slides (Thermo Fisher Scientific) at a density of 1.0×10^6 cells per well and allowed to adhere for 24 hrs. Synoviocytes were incubated with human synoviocyte PRG4 (100 µg/mL) in serum-free DMEM for 24 hours followed by washing with PBS and cell fixation with 4% formalin. Probing was performed using anti- α -SMA antibody (1:100 dilution) or anti-collagen type I antibody (1:200 dilution) (Abcam) at 4°C overnight. Following washing with PBS, cells were incubated with Cy3 goat anti-rabbit IgG antibody (1:200 dilution; Thermo Fisher Scientific) for 1 hr at room temperature in the dark. Following washing with PBS, Alexa Fluor 488-conjugated phalloidin, a filamentous actin (F-actin) probe (1:125 dilution; Thermo Fisher Scientific), was added for 20 min in the dark. Cells were subsequently mounted with DAPI mounting medium (Vector Labs) and viewed under a fluorescent microscope (Nikon E 800).

3.2.10 Basal and TGF- β 1 induced OA synoviocytes proliferation and migration

In sterile 96 well plates, OA synoviocytes (10,000 cells per well) were cultured in serum-free DMEM media and incubated with forskolin (3, 10 and 30µM) $\pm$ TGF- β 1 (1ng/mL) for 48 hours at 37°C. Cell proliferation was determined using the MTT reagent (Sigma). OA synoviocytes (100,000 cells per well) were seeded in 24-well culture plates in DMEM+10% FBS for 72 hours. A 1,000 µL pipette tip was used to perform a scratch in the confluent synoviocyte monolayer.

TGF- β 1 (1ng/mL) stimulation was performed in serum-free DMEM $\pm$ forskolin (10 μ M) for 48 hours. Subsequently, media was aspirated and cells were stained (Cell Biolabs, USA) followed by imaging using all-in-one fluorescence microscope (Keyence, USA). A region of interest (ROI) was defined and the scratch width was measured at multiple locations in the ROI. The mean scratch width was calculated and used to estimate the mean scratch area. Data is presented as the ratio of the scratch areas of the different experimental groups to the scratch area at baseline.

3.2.11 Statistical Analyses

Variables were initially tested for normality. Normally distributed variables were compared using Student's *t*-test for two groups or analysis of variance with Tukey's post-hoc test for more than two groups. Variables that did not satisfy the normality assumption were tested using ANOVA on the ranks. Statistical analysis of gene expression data was performed using Δ Ct values (C_t target gene- C_t GAPDH) for each gene of interest. Significance level was set at 0.05.

3.3 RESULTS

3.3.1 Forskolin treatment increased intracellular cAMP, reduced *ACTA2*, *COL1A1*, *TIMP-1* expression and reduced α -SMA and procollagen type I in TGF- β 1 stimulated OA synoviocytes

A representative dose-response of forskolin is shown in figure 3.1A. Treatment with forskolin (0.01 μ M) did not increase intracellular cAMP while the 0.1, 1 and 10 μ M forskolin treatments resulted in detectable increases in cAMP. Forskolin (10 μ M) increased intracellular cAMP compared to vehicle (*fig. 3.1B*; $p < 0.001$) (n=3 patients). TGF- β 1 induced *ACTA2*, *COL1A1*, *TIMP-1* and *PLOD2* expression (*fig. 3.1C through F*; $p < 0.001$ versus control for the 4 genes) (n=4 patients). Forskolin treatment reduced *ACTA2* (*fig. 3.1C*; $p < 0.001$), *COL1A1* (*fig. 3.1D*; $p < 0.01$), and *TIMP-1* (*fig. 3.1E*; $p < 0.001$) expression compared to TGF- β 1 alone. Forskolin treatment did

not alter TGF- β 1 stimulated *PLOD2* expression (*fig. 3.1F*; $p=0.833$). Forskolin did not alter basal *COL1A1* ($p=0.623$), *TIMP-1* ($p=0.802$) or *PLOD2* ($p=0.752$) expression. In contrast, forskolin reduced basal *ACTA2* expression ($p=0.018$).

A Western Blot and semi-quantitative analysis of α -SMA using GAPDH as a loading control are shown in *fig. 3.1G* and *fig. 3.1H*, *respectively*. TGF- β 1 increased α -SMA protein in OA synoviocytes compared to control (*fig. 3.1H*; $p<0.001$) ($n=6$ patients). Forskolin reduced TGF- β 1 stimulated α -SMA production ($p=0.013$). Forskolin alone did not alter basal α -SMA content ($p=0.660$). Representative confocal images of TGF- β 1-treated OA synoviocytes $\pm$ forskolin is shown in *figure 3.1I*. Control OA synoviocytes exhibited a positive α -SMA staining, and the appearance of a myofibroblast-like phenotype in a number of cells. TGF- β 1 treatment resulted in stronger α -SMA staining and the appearance of myofibrils and this was markedly reduced with forskolin co-treatment. TGF- β 1 increased procollagen type I protein compared to control (*fig. 3.1J*; $p<0.01$) ($n=3$ patients). Forskolin reduced TGF- β 1 linked procollagen type I production ($p=0.015$), and did not alter basal procollagen type I ($p=0.991$).

3.3.2 Forskolin treatment enhanced HA secretion and modulated HAS isoform gene expression and *HAS1* knockdown attenuated forskolin's effect on HA production in TGF- β 1 stimulated OA synoviocytes

HA concentrations were higher in TGF- β 1 treated OA synoviocytes and forskolin-treated OA synoviocytes compared to untreated controls (*fig. 3.2A*; $p<0.001$ for both comparisons) ($n=4$ patients). HA concentrations in the TGF- β 1 + forskolin group were higher than HA concentrations in the TGF- β 1 or forskolin alone groups ($p<0.001$ for both comparisons). TGF- β 1 induced *HAS1* (*fig. 3.2B*; $p<0.001$) and *HAS2* (*fig. 3.2C*; $p<0.001$) with no effect on *HAS3* (*fig. 3.2D*; $p=0.719$) expression in OA synoviocytes ($n=4$ patients). Forskolin treatment upregulated basal *HAS1*, *HAS2* and *HAS3* expression ($p<0.001$ for all comparisons). Combined treatment of TGF- β 1 and

forskolin increased *HAS1* expression over TGF- β 1 alone (*fig. 3.2B*; $p=0.026$). In contrast, *HAS2* expression in the TGF- β 1 + forskolin group was lower than *HAS2* expression in the TGF- β 1 group (*fig. 3.2C*; $p=0.024$).

The total cellular *HAS1* content was higher in TGF- β 1 ($p=0.021$) and TGF- β 1 + forskolin ($p<0.01$) treatments compared to control (*fig. 3.2E*) ($n=4$ patients). *HAS1* content was not different between TGF- β 1 + forskolin and TGF- β 1 alone groups ($p=0.971$). Similarly, there was no difference in *HAS1* between forskolin and control groups ($p=0.303$). *HAS1* expression was reduced by approximately 68% in OA synoviocytes transfected with *HAS1* siRNA (*fig. 3.2F*; $p<0.01$) ($n=4$ OA patients). HA concentrations in TGF- β 1 stimulated *HAS1* knockdown OA synoviocytes were not different from unstimulated *HAS1* knockdown OA synoviocytes (*fig. 3.2G*; $p=0.962$) ($n=4$ patients). Similarly, HA concentrations in TGF- β 1 + forskolin treated *HAS1* knockdown OA synoviocytes were not different from HA concentrations in TGF- β 1 treated *HAS1* knockdown OA synoviocytes ($p=0.514$). Finally, HA concentrations in TGF- β 1 + forskolin treated OA synoviocytes were higher than HA concentrations in TGF- β 1 + forskolin treated *HAS1* knockdown OA synoviocytes ($p<0.001$).

3.3.3 Forskolin treatment enhanced *PRG4* expression and secretion in TGF- β 1 stimulated OA synoviocytes

TGF- β 1 induced *PRG4* expression (*fig. 3.3A*; $p<0.001$) and increased *PRG4* production by OA synoviocytes (*fig. 3.3B*; $p<0.01$) ($n=4$ patients). Forskolin did not alter basal *PRG4* expression (*fig. 3.3A*; $p=0.063$) or *PRG4* production (*fig. 3.3B*; $p=0.996$) in OA synoviocytes. *PRG4* expression in the TGF- β 1 + forskolin group was higher than TGF- β 1 alone (*fig. 3.3A*; $p=0.037$). Correspondingly, *PRG4* concentrations were higher in the TGF- β 1 + forskolin group compared to the TGF- β 1 group (*fig. 3.3B*; $p=0.031$).

3.3.4 Impact of PRG4 and HA treatments on *ACTA2* and *COL1A1* expression and α -SMA and procollagen type I in TGF- β 1 stimulated OA synoviocytes

ACTA2 expression was lower in the TGF- β 1 + PRG4 group compared to TGF- β 1 alone (fig. 3.3C; $p < 0.001$) (n=4 patients). Similarly, *ACTA2* expression in the TGF- β 1 + HA and TGF- β 1 + PRG4 + HA groups was lower than *ACTA2* expression in TGF- β 1 alone ($p < 0.001$ for both comparisons). *COL1A1* expression was lower in the TGF- β 1 + PRG4 group compared to TGF- β 1 + HA ($p < 0.01$) or TGF- β 1 alone ($p < 0.001$) (fig. 3.3D) (n=4 OA patients). In contrast, HA treatment did not alter TGF- β 1 induced *COL1A1* expression ($p = 0.897$). *COL1A1* expression in the TGF- β 1 + PRG4 + HA group was lower than TGF- β 1 ($p < 0.001$) and TGF- β 1 + HA ($p < 0.01$) groups.

A Western Blot and semi-quantitative analysis of α -SMA using GAPDH as a loading control are shown in 3.3E and 3.3F, respectively. α -SMA content was lower in TGF- β 1 + PRG4 (fig. 3.3F; $p < 0.01$) and in TGF- β 1 + HA ($p < 0.01$) compared to TGF- β 1 alone (n=4 patients). Procollagen type I content was lower in TGF- β 1 + PRG4 compared to TGF- β 1 alone (fig. 3.3G; $p < 0.01$) (n=4 patients). There was no difference in procollagen type I content between TGF- β 1 + HA and TGF- β 1 groups ($p = 0.059$).

3.3.5 A cell permeable cAMP analog treatment reduced *ACTA2* and *COL1A1* expression and enhanced HA and PRG4 secretion in TGF- β 1 stimulated OA synoviocytes

TGF- β 1 induced *ACTA2* and *COL1A1* expression in OA synoviocytes (fig. 3.4A and 3.4B; $p < 0.001$ against control for both genes). 8-bromo cAMP (100 μ M) treatment did not significantly alter TGF- β 1 induced *ACTA2* and *COL1A1* expression ($p > 0.05$ for both comparisons). In contrast, 8-bromo cAMP (500 μ M) treatment reduced *ACTA2* ($p < 0.001$) and *COL1A1* ($p < 0.001$) expression in TGF- β 1 stimulated OA synoviocytes (n=3 OA patients). HA and PRG4 media concentrations in TGF- β 1 + 8-bromo cAMP (500 μ M) group were significantly higher than

corresponding concentrations in TGF- β 1 only group (fig. 3.4C and 3.4D; $p<0.001$ for both comparisons) (n=3 OA patients).

3.3.6 *ACTA2*, *COL1A1* and *TIMP-1* expression was higher in *Prg4*^{-/-} synovial tissues and human synoviocyte PRG4 treatment reduced α -SMA and collagen type I staining in *Prg4*^{-/-} synoviocytes

Expression of *ACTA2* ($p=0.021$), *COL1A1* ($p<0.001$) and *TIMP-1* ($p<0.01$) was higher in *Prg4*^{-/-} synovia compared to *Prg4*^{+/+} synovia (fig. 3.5A). In contrast, *PLOD2* expression was lower in *Prg4*^{-/-} tissues compared to *Prg4*^{+/+} tissues ($p<0.01$).

Merged images of α -SMA and collagen type I stained *Prg4*^{+/+} and *Prg4*^{-/-} synoviocytes is shown in figure 3.5B. We observed strong α -SMA and collagen type I staining in *Prg4*^{-/-} synoviocytes. α -SMA staining co-localized with F-actin staining. In contrast, there was no detected α -SMA or collagen type I staining in *Prg4*^{+/+} synoviocytes. PRG4 treatment reduced α -SMA and collagen type I staining in *Prg4*^{-/-} synoviocytes.

3.3.7 Forskolin reduced TGF- β 1 induced OA synoviocyte proliferation and migration

Forskolin (3 μ M and 10 μ M) treatments did not alter basal OA synoviocyte proliferation (fig. 3.6A; $p=0.891$ and $p=0.117$) (n=4 patients). In contrast, the 30 μ M treatment increased basal OA synoviocyte proliferation compared to untreated control ($p<0.01$). TGF- β 1 stimulated OA synoviocyte proliferation (fig. 3.6B; $p<0.001$) (n=4 patients). OA synoviocyte proliferation in the TGF- β 1 + forskolin (30 μ M) group was lower than TGF- β 1 alone ($p<0.001$), TGF- β 1 + forskolin (3 μ M) ($p<0.01$) or TGF- β 1 + forskolin (10 μ M) ($p<0.01$). There was no difference in cell proliferation between TGF- β 1 + forskolin (10 μ M) and TGF- β 1 alone ($p=0.063$). Representative wound scratch images are shown in figure 3.6C. TGF- β 1 enhanced OA synoviocyte migration (fig. 3.6D; $p<0.01$) (n=4 patients). OA synoviocyte migration in the TGF- β 1 + forskolin (10 μ M) group

was lower than in the TGF- β 1 only group ($p<0.01$). There was no difference in cell migration between forskolin treated and untreated OA synoviocytes ($p=0.887$).

3.4 DISCUSSION

In this paper, we show that TGF- β 1 resulted in excess collagen type I production, induction of *TIMP-1* and *PLOD2* expression and α -SMA upregulation, together with stimulating OA synoviocyte migration and proliferation. Forskolin, by virtue of its ability to generate cAMP, reduced collagen production and blunted TIMP-1 expression while inhibiting synoviocyte proliferation and migration. *PLOD2* induction in osteoarthritic synoviocytes is consistent with its established role in mediating synovial collagen crosslinking [181, 182]. Forskolin did not alter *PLOD2* expression, which may be related to the TGF- β 1's signaling pathways. Remst *et al* have shown that in OA synoviocytes, an ALK 1/2/3/6 inhibitor completely blocked TGF- β 1 induced collagen type I expression whereas TGF- β 1 induced *PLOD2* expression was only slightly reduced [183].

OA synoviocytes proliferate in response to various mitogenic stimuli [155, 184, 185]. In our experiments, TGF- β 1 induced cell proliferation with a magnitude comparable to what has been previously reported [184]. Forskolin, at the treatment level that inhibited migration, exhibited a marginal antiproliferative effect and a higher concentration was needed to observe significant antagonism of TGF- β 1's mitogenic effect. This might be due to a low proliferative capacity of OA synoviocytes. In the absence of TGF- β 1, forskolin acted as a mitogen to produce a low, yet significant, stimulation of proliferation. Forskolin directly binds to AC and generates cAMP from ATP [186]. The increase in cAMP results in activation of protein kinase A (PKA) dependent and independent pathways [186, 187]. In the presence of growth factors, forskolin activates cAMP-

dependent PKA which interferes with Raf-1 activation and signaling to blunt cell proliferation [67]. PKA also activates CREB, which can compete for cofactors with SMAD-mediated transcription stimulated by TGF- β 1[177]. In the absence of growth factor, PKA-dependent and independent pathways stimulate cAMP-mediated cell proliferation [188].

We measured α -SMA in OA synoviocytes from human patients and forskolin addition consistently attenuated TGF- β 1-stimulated α -SMA expression. α -SMA is a specific marker of myofibroblasts [189, 190]. Myofibroblasts are effector cells in fibrosis that possess enhanced ability to produce collagen, proliferate and migrate [189, 190]. We have shown that forskolin treatment appeared to markedly reduce TGF- β 1's induced myofibroblast-like phenotype in OA synoviocytes. The causal role that myofibroblasts may play in synovial fibrosis is understudied and unclear. Steenvoorden *et al* reported that α -SMA staining was only found in blood vessels in synovia from healthy individuals [191]. Interestingly, TGF- β 1 stimulation of normal synoviocytes increased collagen type I expression with no effect on α -SMA expression or production [191]. Matthey *et al* have shown that TGF- β 1 or IL-4 treatments trigger differentiation of OA synoviocytes into myofibroblast-like cells, characterized by α -SMA expression *in vitro* [166]. Evidence relating myofibroblasts to changes occurring in joint fibrosis was reported by Sasabe *et al* [192]. Using a rat knee contracture model, myofibroblasts expressing α -SMA were detected as early as 1 week from joint immobilization and this was associated with increased collagen type I expression and joint capsule fibrosis [192]. We have also detected α -SMA protein in *Prg4*^{-/-} synoviocytes with no α -SMA signal in normal murine synoviocytes. The positive α -SMA signal in *Prg4*^{-/-} knockout synoviocytes is associated with phenotypical changes in the synovium including synovial lining thickening and enhanced synoviocyte proliferation [81].

TGF- β 1 induced *HAS1* and *HAS2* expression with no effect on *HAS3*, and enhanced HA secretion by OA synoviocytes. Synoviocytes contain higher levels of *HAS1* message compared to *HAS2* with *HAS3* being the least abundant [193]. Earlier reports are in agreement with our finding that TGF- β 1 stimulation of arthritic synoviocytes resulted in a higher HAS1/HAS2 transcript ratio above control level and that in turn resulted in higher extracellular HA levels [184, 193]. Our data suggests that the majority of TGF- β 1 linked HA secretion is mediated by HAS1, as HAS1 knockdown diminished extracellular HA concentrations. Co-treatment with forskolin increased the HAS1/HAS2 transcript ratio above the corresponding TGF- β 1 ratio with greater HA secretion over 24 hours. The increase in HAS1 mRNA in forskolin-treated synoviocytes did not translate to increased HAS1 cellular pool. This might be related to the rate at which the message is being translated. Furthermore, the majority of HAS1 cellular fraction is inactive and is found in the cytoplasm either diffused or partially co-localized with the Golgi apparatus, whereas the plasma membrane-bound fraction is small and is catalytically active [178]. Therefore, the possibility that the newly synthesized HAS1 enzyme, in response to forskolin treatment, could have been trafficked to the membrane resulting in increasing the rate of HA synthesis could not be ruled out. Assessing the impact of forskolin on membrane-bound HAS1 level and activity was technically challenging and was not feasible to perform. Our data should also be considered in the context that other factors *e.g.* post-translational modifications, availability of precursors and regulation of HAS activity will likely contribute to the amount of HA secreted by the OA synoviocytes[193] .

Increasing intracellular cAMP resulted in increasing PRG4 expression and production by OA synoviocytes in the setting of TGF- β 1 stimulation. This contextual effect is due to CREB stimulation which was previously shown to enhance PRG4 production by superficial zone articular chondrocytes [194]. PRG4 is a mucin-like glycoprotein synthesized by synoviocytes and

superficial zone articular chondrocytes with a heavily glycosylated central domain and an N- and C-termini [195]. The mouse *Prg4* gene is highly homologous to the human *Prg4* gene [196]. Human and mouse *Prg4* genes each consist of 12 exons and the N- and C-termini are highly conserved across species [196]. PRG4 exists in the synovial fluid in monomeric or multimeric forms and functions as a boundary lubricant [195, 197]. PRG4 binds to CD44, the HA receptor, and exerts an anti-inflammatory effect in OA synoviocytes [86, 155]. PRG4 also acts in an autocrine manner to regulate OA synoviocyte proliferation [155]. We have found that PRG4 and HA had equivalent efficacy in reducing α -SMA content in osteoarthritis synoviocytes. However, PRG4, at a physiologically-relevant concentration [150], was more efficacious than HA in reducing collagen I expression and production, indicative of a potential role in antagonizing profibrotic alterations in synovial tissues. This function is likely related to its interaction with the CD44 receptor, given that HA reduced synovial fibrosis in a CD44-mediated manner [92]. PRG4 reduced collagen I and α -SMA staining in *Prg4*^{-/-} synoviocytes. The link between PRG4 expression and synovial fibrosis is further illustrated by the upregulation of collagen type I, TIMP-1 and α -SMA in the *Prg4*^{-/-} synovium. As laying excess collagen type I is a prominent feature in synovial fibrosis, the strong immunocytochemical staining for collagen type I in *Prg4*^{-/-} synoviocytes, coupled with other synovial changes, support a fibrotic *Prg4*^{-/-} synovium. We did not include human normal synoviocytes in our study design. Furthermore, we did not examine the efficacy of forskolin or PRG4 in an *in-vivo* model of synovial fibrosis.

In summary, our data demonstrate that forskolin; a diterpene produced by the roots of the Indian plant *Coleus forskohlii* [186], increases intracellular cAMP levels and produces an antifibrotic effect in OA synoviocytes. Increasing intracellular cAMP levels directly via treatment with a cell permeable cAMP analog recapitulated the antifibrotic effect of forskolin. Forskolin reduces

collagen type I expression and procollagen type I production and inhibits TGF- β 1 linked fibroblast migration and proliferation. Forskolin also increased HA and PRG4 secretion by OA synoviocytes; an effect that may contribute to its overall antifibrotic efficacy. Approaches that increase cAMP levels in synoviocytes can promote an antifibrotic phenotype and may be a novel approach for slowing the progression of synovial fibrosis in OA.

3.5 GRANTS

This work is supported by R01AR067748 to KE and GJ.

3.6 DISCLOSURES

Authors MQ, LZ and RO have nothing to disclose. GJ authored patents related to use of recombinant PRG4 and holds equity in Lubris LLC, MA, USA. KE co-authored patent applications related to use of recombinant PRG4.

3.7 AUTHOR CONTRIBUTIONS

Authors MQ, LZ and KE carried out the experiments and participated in data analysis. GJ participated in study design and critical interpretation of results. KE and RO conceived the study and participated in data analysis and interpretation. All authors participated in drafting and critical evaluation of the manuscript. All authors have read and approved the final version of the manuscript.

3.8 ACKNOWLEDGEMENTS

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3.9 FIGURES

Fig. 3.1 Impact of forskolin (FsK) treatment on intracellular cyclic adenosine monophosphate (cAMP) levels, basal and transforming growth factor beta 1 (TGF- β 1)-induced alpha smooth muscle actin (*ACTA2*), collagen I (*COL1A1*), tissue inhibitor of metalloproteinase 1 (*TIMP-1*) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) gene expression and alpha smooth muscle action (α -SMA) and procollagen type I production in osteoarthritic (OA) synoviocytes. TGF- β 1 (1ng/ml) stimulation of OA-FLS was performed for 24 hours in all experiments except α -SMA immunocytochemical staining (stimulation was performed using 1 ng/ml TGF- β 1 for 48 hours). Data is presented as the mean $\pm$ S.D. of experiments utilizing OA synoviocytes from different patients. * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$.

A. Representative dynamic change in intracellular cAMP levels in OA synoviocytes following treatment with FsK (0.01, 0.1, 1 and 10 μ M). FsK treatment (0.1, 1 and 10 μ M) resulted in detectable cAMP levels in OA synoviocytes. The cAMP signal was detected using a cAMP-specific sensor. **B.** cAMP levels were elevated in FsK (10 μ M)-treated OA synoviocytes (n=3 patients). **C.** FsK treatment (1 and 10 μ M) reduced TGF- β 1 induced *ACTA2* expression (n=4

patients). **D.** Fsk treatment (10 μ M) reduced TGF- β 1 induced *COL1A1* expression (n=4 patients). **E.** Fsk treatment (10 μ M) reduced TGF- β 1 induced *TIMP-1* expression (n=4 patients). **F.** Fsk treatment (10 μ M) did not alter TGF- β 1 induced *PLOD2* expression (n=4 patients). **G.** Western Blot of α -SMA in control, TGF- β 1, TGF- β 1 + Fsk and Fsk-treated OA synoviocytes. GAPDH was used as loading control. **H.** Semi-quantitative densitometry analysis of α -SMA normalized to GAPDH and expressed as ratio to control in cell extracts of control, TGF- β 1, TGF- β 1 + Fsk and Fsk-treated OA synoviocytes. Fsk (10 μ M) treatment reduced TGF- β 1 linked increase in α -SMA in OA synoviocytes (n=6 patients). **I.** Fsk (10 μ M) treatment reduced α -SMA staining and myofibroblast-like phenotype in TGF- β 1 stimulated OA synoviocytes. **J.** Procollagen type I content in cell extracts of control, TGF- β 1, TGF- β 1 + Fsk and Fsk-treated OA synoviocytes. Data was normalized to total protein content. Fsk (10 μ M) treatment reduced TGF- β 1 linked increase in procollagen type I content in OA synoviocytes (n=3 patients).

Fig. 3.2 Impact of forskolin (FsK; 10 μ M) treatment on basal and transforming growth factor beta 1 (TGF- β 1)-induced hyaluronan (HA) production, expression of hyaluronan synthase isoforms 1, 2, and 3 (*HAS1*, *HAS2* and *HAS3*) and the role of *HAS1* in mediating TGF- β 1 and FsK-linked HA production in osteoarthritic (OA) synoviocytes. Data is presented as the mean $\pm$ S.D. of experiments utilizing OA synoviocytes from 4 patients. * p <0.001; ** p <0.01; *** p <0.05; n.s.: non significant.

A. Hyaluronan concentrations in control, TGF- β 1, TGF- β 1 + Fsk, and Fsk-treated OA synoviocytes. **B.** Fsk treatment enhanced TGF- β 1 induced *HAS1* expression. **C.** Fsk treatment

reduced TGF- β 1 induced *HAS2* expression. **D.** FsK treatment increased basal *HAS3* expression in OA synoviocytes. **E.** HAS1 protein content in cell extracts of control, TGF- β 1, TGF- β 1 + FsK and FsK-treated OA synoviocytes. Data was normalized to total protein content. There was no difference in HAS1 protein between TGF- β 1 and TGF- β 1 + FsK treatments. **F.** HAS1 expression was reduced in HAS1 siRNA-treated OA synoviocytes compared to untreated control and negative control siRNA (NC siRNA)-treated OA synoviocytes. **G.** FsK and/or TGF- β 1 treatments did not significantly change hyaluronan production following *HAS1* knockdown in OA synoviocytes.

Fig. 3.3 Impact of forskolin (FsK; 10 μ M) treatment on basal and transforming growth factor beta 1 (TGF- β 1) induced proteoglycan-4 (PRG4) gene expression and production by osteoarthritic (OA) synoviocytes and efficacy of human synoviocyte PRG4 (100 μ g/ml) and hyaluronan (HA) (100 μ g/ml) in modulating TGF- β 1 induced expression and production of alpha smooth muscle actin and collagen type I in OA synoviocytes. Data is presented as the mean $\pm$ S.D. of experiments utilizing OA synoviocytes from 4 patients. * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$.

A. TGF- β 1 increased PRG4 expression and FsK treatment enhanced TGF- β 1's effect. **B.** FsK treatment enhanced TGF- β 1 linked PRG4 production by OA synoviocytes. **C.** PRG4 and/or HA treatments reduced TGF- β 1 induced alpha smooth muscle actin gene (*ACTA2*) expression in OA synoviocytes. **D.** PRG4 treatment reduced TGF- β 1 induced collagen type I (*COL1A1*) gene expression in OA synoviocytes. **E.** Western Blot of α -SMA in control, TGF- β 1, TGF- β 1 + PRG4 (100 μ g/ml) and TGF- β 1 + HA (100 μ g/ml) treated OA synoviocytes. GAPDH was used as loading control. **F.** Semi-quantitative densitometry analysis of α -SMA normalized to GAPDH and

expressed as ratio to control in cell extracts of control, TGF- β 1, TGF- β 1 + PRG4 and TGF- β 1 + HA treated OA synoviocytes. PRG4 and HA treatments reduced TGF- β 1 linked increase in α -SMA in OA synoviocytes. **G.** Procollagen type I content in cell extracts of control, TGF- β 1, TGF- β 1 + PRG4 and TGF- β 1 + HA-treated OA synoviocytes. Data was normalized to total protein content. PRG4 treatment reduced TGF- β 1 linked increase in procollagen type I content in OA synoviocytes.

Fig. 3.4 Impact of 8-bromo cAMP (8-Br-cAMP; 100 and 500 μ M) treatment on basal and transforming growth factor beta 1 (TGF- β 1)-induced alpha smooth muscle actin (*ACTA2*) and collagen I (*COL1A1*) expression and hyaluronan (HA) and proteoglycan-4 (PRG4) secretion in osteoarthritic synoviocytes. Data is presented as the mean $\pm$ S.D. of experiments utilizing OA synoviocytes from 3 patients. $*p < 0.001$. **A.** 8-Br-cAMP (500 μ M) reduced TGF- β 1 linked *ACTA2* expression in OA synoviocytes. **B.** 8-Br-cAMP (500 μ M) reduced TGF- β 1 linked *COL1A1* expression in OA synoviocytes. **C.** 8-Br-cAMP (500 μ M) increased HA secretion in TGF- β 1stimulated OA synoviocytes. **D.** 8-Br-cAMP (500 μ M) increased PRG4 secretion in TGF- β 1stimulated OA synoviocytes.

Fig. 3.5 Gene expression of alpha smooth muscle actin (*ACTA2*), collagen type I (*COL1A1*), tissue-inhibitor of metalloproteinase-1 (*TIMP-1*) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) in synovial tissues isolated from *Prg4*^{+/+} and *Prg4*^{-/-} mice and immunocytochemical staining of alpha smooth muscle actin (α -SMA) and collagen type I in *Prg4*^{+/+} and *Prg4*^{-/-} synoviocytes and impact of human synoviocyte PRG4 treatment. * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$.

A. *ACTA2*, *COL1A1* and *TIMP-1* expression in *Prg4*^{-/-} synovial tissues was higher than *Prg4*^{+/+} synovial tissues. *PLOD2* expression in *Prg4*^{-/-} synovial tissues was lower than *Prg4*^{+/+} synovial tissues. Each group contained 5 samples with each sample generated by pooling synovial tissues from 3 mice.

B. Merged images depicting α -SMA and collagen type I protein immunostaining in isolated *Prg4*^{+/+} synoviocytes and *Prg4*^{-/-} synoviocytes (bright orange) and counterstained with F-actin [198] and DAPI (blue). α -SMA and collagen type I staining was detected in *Prg4*^{-/-} synoviocytes (white arrows) and no staining was detected in *Prg4*^{+/+} synoviocytes. α -SMA and collagen type I staining intensities were reduced by human synoviocyte PRG4 treatment for 24 hours. Scale = 50 μ m.

Fig. 3.6 Impact of forskolin (FsK) treatment on basal and transforming growth factor beta 1 (TGF-β1) induced proliferation and migration of osteoarthritic (OA) synoviocytes. Data is presented as the mean $\pm$ S.D. of experiments utilizing OA synoviocytes from 4 patients. $*p<0.001$; $**p<0.01$; $***p<0.05$. Scale = 1,000 μ M. **A.** FsK (30 μ M) treatment enhanced basal OA synoviocyte proliferation. **B.** FsK (30 μ M) treatment reduced TGF-β1-induced OA synoviocyte proliferation. **C.** Representative images showing baseline and 48-hour basal, TGF-β1, TGF-β1 + FsK and FsK alone induced OA synoviocyte migration using an *in vitro* scratch assay. **D.** FSK (10 μ M) treatment reduced TGF-β1 stimulated OA synoviocyte migration.

4 CHAPTER 4

Proteoglycan-4 Regulates Fibroblast to Myofibroblast Transition and Expression of Fibrotic Genes in the Synovium

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ABSTRACT

Background: Synovial tissue fibrosis is common in advanced OA with features including presence of stress fiber-positive myofibroblasts and deposition of cross-linked collagen type-I. An antifibrotic effect in OA synoviocytes was associated with PRG4 secretion and native synovial PRG4 reduced collagen content in OA synoviocytes. PRG4 is a ligand of the CD44 receptor. Our objective was to examine the role of PRG4-CD44 interaction in regulating synovial tissue fibrosis *in vitro* and *in vivo*.

Methods: OA synoviocytes were treated with TGF- β $\pm$ PRG4 for 24 hours and α -SMA content was determined using immunofluorescence. Rhodamine labeled rhPRG4 was incubated with OA synoviocytes $\pm$ anti-CD44 or isotype control antibodies and cellular uptake of rhPRG4 was determined following a 30-min incubation and α -SMA expression following a 24-hour incubation. HEK-TGF- β cells were treated with TGF- β $\pm$ rhPRG4 and Smad3 phosphorylation was determined using immunofluorescence and TGF- β /Smad pathway activation was determined colorimetrically. We probed for stress fibers and focal adhesions (FAs) in TGF- β treated murine fibroblasts and fibroblast migration was quantified $\pm$ rhPRG4. Synovial expression of fibrotic markers: α -SMA, collagen type-I and PLOD2 in *Prg4* gene trap (*Prg4^{GT}*) and recombined *Prg4^{GTR}* animals was studied at 2 and 9 months of age. Synovial expression of α -SMA and PLOD2 was determined in 2-months old *Prg4^{GT/GT}&Cd44^{-/-}* and *Prg4^{GTR/GTR}&Cd44^{-/-}* animals.

Results: PRG4 reduced α -SMA content in OA synoviocytes ($p<0.001$). rhPRG4 was internalized by OA synoviocytes *via* CD44 and CD44 neutralization attenuated rhPRG4's antifibrotic effect ($p<0.05$). rhPRG4 reduced pSmad3 signal in HEK-TGF- β cells ($p<0.001$) and TGF- β /Smad

pathway activation ($p<0.001$). rhPRG4 reduced the number of stress fiber-positive myofibroblasts, FAs mean size, and cell migration in TGF- β treated NIH3T3 fibroblasts ($p<0.05$). rhPRG4 inhibited fibroblast migration in a macrophage and fibroblast co-culture model without altering active or total TGF- β levels. Synovial tissues of 9-months old *Prg4*^{GT/GT} animals had higher α -SMA, collagen type-I and PLOD2 ($p<0.001$) content and *Prg4* re-expression reduced these markers ($p<0.01$). *Prg4* re-expression also reduced α -SMA and PLOD2 in CD44-deficient mice.

Conclusion: PRG4 is an endogenous antifibrotic modulator in the joint and its effect on myofibroblast formation is mediated by CD44, but CD44 is not required to demonstrate an antifibrotic effect *in vivo*.

Keywords: Proteoglycan-4, CD44, SMA, synovial fibrosis, myofibroblast

4.1 INTRODUCTION

Osteoarthritis (OA) is the most prevalent type of musculoskeletal disorder in patients 60 years or older [199-201]. OA is a leading cause of pain and disability and patients with symptomatic knee OA are at an increased risk of all-cause mortality [202-204]. The etiology of OA is complex where joint trauma, obesity and genetics, among other risk factors, contribute to disease development [205-208]. OA affects the whole joint with manifestations that include degeneration of articular cartilage and the meniscus, abnormal bone remodeling, and synovial inflammation [160, 209]. Macroscopic evidence of synovial inflammation or synovitis is a common finding in up to 74% of patients with knee OA of different grades and 95% of patients with moderate to severe OA [112, 210, 211]. OA synovitis is likely caused by an innate immune response, mediated by the toll-like receptors 2 and 4 (TLR2 and TLR4) in the synovium, and resultant expression of inflammatory cytokines, chemokines and matrix degrading enzymes [140, 212-215]. The extent of synovitis is a strong predictor of OA progression across multiple studies, and therefore treating synovial inflammation is a potentially important target for therapeutic intervention, especially during the early stage of OA [112, 157, 216].

The normal synovium is composed of a cellular intimal layer and a subintimal connective tissue layer [112, 115]. The intimal layer is usually 1-4 cells in thickness with two types of cells; type A macrophages and type B synovial fibroblasts, with macrophages making up a smaller percentage of cells in the intimal layer of the healthy synovium [115]. Synovial fibroblasts synthesize hyaluronic acid (HA), a glycosaminoglycan, and proteoglycan-4 (PRG4), a mucinous glycoprotein, which are major components of synovial fluid (SF) with important roles in joint lubrication [195, 217]. In synovitis, synovial membranes become hyperplastic with increased accumulation of macrophages and other inflammatory cells, along with subintimal fibrosis and

angiogenesis [112, 160, 161]. Synovial fibrosis can be considered a maladaptive healing response to chronic synovitis which is characterized by upregulation of collagen type-I expression, fibroblast transition to a myofibroblast-like phenotype through *de-novo* expression of alpha smooth muscle actin (α -SMA) and enhanced migratory behavior, and upregulation of procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) leading to accumulation of cross-linked collagen in the synovium [164, 218]. These pathological changes are induced in the OA synovium by transforming growth factor beta (TGF)- β 1 and its associated signaling pathways [162, 183]. Synovial fibrosis independently contributes to joint pain and stiffness observed in advanced OA [161, 219].

In a recent study, we demonstrated that increasing intracellular adenosine 3', 5'-cyclic monophosphate (cAMP), using forskolin, reduced collagen type-I, PLOD2 and α -SMA expression in TGF- β stimulated osteoarthritic fibroblast-like synoviocytes (OA FLS) and reduced fibroblast to myofibroblast transition, as evidenced by blocking stress fiber formation [220]. Associated with this antifibrotic effect was an increase in HA and PRG4 production by OA FLS, whereby native synovial human PRG4 reduced procollagen type-I level in OA FLS [220]. Furthermore, synoviocytes from *Prg4* null mice displayed more extensive collagen type-I and α -SMA immunostaining compared to synoviocytes from *Prg4* competent mice [220]. In a separate study, we have also shown that PRG4 is a ligand for the HA receptor, CD44 [154]. We have also reported that PRG4-CD44 interaction inhibited interleukin-1 beta (IL-1 β) induced OA FLS proliferation and expression of matrix degrading enzymes [155], via the inhibition of nuclear factor kappa b (NF- κ B) nuclear translocation mediated by blocking inhibitory kappa b (I κ B) degradation [155].

It remains unknown whether PRG4 has a role in regulating fibroblast to myofibroblast transition and associated cell migration in the fibrotic synovium. Furthermore, it is yet to be determined whether PRG4 regulates synovial fibrosis *in vivo* and whether this role is due to its interaction with the CD44 receptor. Using recombinant human proteoglycan-4 (rhPRG4), we aimed to study the role of PRG4 in regulating fibroblast to myofibroblast transition and modulating fibroblast migration in response to exogenous TGF- β or co-incubation with lipopolysaccharide (LPS) stimulated macrophages. We also studied the role of PRG4-CD44 interaction, and more specifically CD44-mediated cellular uptake of PRG4, in the regulation of myofibroblast formation *in vitro* and progression of synovial fibrosis *in vivo*. We hypothesized that PRG4 regulates fibroblast to myofibroblast transition and prevents synovial fibrosis in a CD44-dependent manner.

4.2 METHODS

4.2.1 Impact of PRG4 and HA treatments on ACTA2 expression, α -SMA immunostaining, and stress fiber formation in osteoarthritic fibroblast-like synoviocytes (OA FLS) and role of CD44 in mediating the effect of rhPRG4 in TGF- β stimulated OA FLS

OA FLS (Cell Applications, USA) were isolated from synovial tissues from de-identified patients undergoing knee replacement surgery (n=4; 2 males and 2 females; 62-69 years old). Cells were received in their second passage and were cultured as previously described [213]. OA FLS were used between the 3rd and 6th passages to avoid alterations in gene expression pattern and cell proliferation rate [176]. OA FLS (300,000 cells/well) in serum-free DMEM media were treated with recombinant human TGF- β 1 (R&D Systems, USA) (1ng/mL) $\pm$ native human synovial PRG4 (apparent mol mass 280 kDa as a monomer; 100 μ g/mL) [179] or high molecular weight hyaluronic acid (HA; ~1,200 kDa; R&D Systems; 100 μ g/mL) for 24 hours. RNA isolation, cDNA synthesis and quantitative PCR were performed as previously described [220]. The cycle threshold (Ct) value of α -SMA (*ACTA2*) was normalized to the Ct value of *GAPDH* in the same sample, and the

relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method [134]. All primers and probes utilized in our study are commercially available (Thermo Fisher Scientific, USA).

Assessment of α -SMA content in OA FLS was conducted using immunofluorescence and determination of corrected total cell fluorescence (CTCF) using a Nikon E600 fluorescence microscope. OA FLS (200,000 cells/well) were cultured on collagen type I-coated 22 mm glass coverslips for 48 hours in DMEM medium + 10% fetal bovine serum (FBS). Cells were treated with TGF- β 1 (1ng/mL) $\pm$ PRG4 or HA (100 μ g/mL for both treatments) for 48 hours in serum-free DMEM medium. Subsequently, cells were fixed in 10% neutral buffered formalin for 15 min and washed twice with phosphate buffered saline (PBS). Cells were permeabilized using 0.01% Triton X-100 in PBS and blocked using 2% bovine serum albumin (BSA; Sigma Aldrich, USA) in PBS for 2 hours at room temperature. Probing for α -SMA was performed using FITC-conjugated anti- α -SMA antibody (1:100 dilution; Abcam, USA) and counterstained using Alexa Fluor 594-conjugated anti- α -tubulin antibody (1:100 dilution: Abcam) overnight at 4°C. Following washing with PBS, cells were mounted on DAPI mounting shield (Abcam) and CTCF was quantified using 4 different fields per slide and mean CTCF was calculated. Presence of stress fibers in OA FLS was also evaluated.

Recombinant human PRG4 (rhPRG4; apparent mol mass 240 kDa) is an endotoxin-free full-length product produced by CHO-M cells (Lubris, Framingham, USA) [221]. Rhodamine labeling of rhPRG4 was performed using the Pierce NHS-Rhodamine Antibody Labeling Kit (Thermo Fisher Scientific). OA FLS (200,000 cells/well) were cultured on collagen type-I coated cover slips and incubated with rhodamine-rhPRG4 (25 μ g/mL) $\pm$ anti-CD44 or isotype control (IC) antibodies (2 μ g/mL for both antibodies; Abcam) for 30 minutes. Cells were pre-incubated with

the antibodies for 1-hour prior to rhPRG4 addition. Subsequently, cells were washed twice with PBS and mounted on DAPI mounting media and CTCF was quantified as described above. In another set of experiments, OA FLS (300,000 cells/well) were treated with TGF- β 1 (1ng/mL) $\pm$ rhPRG4 (100 μ g/mL) $\pm$ anti-CD44 or IC antibodies (2 μ g/mL for both antibodies) for 24 hours followed by determination of *ACTA2* expression as described above.

4.4.2 CD44-dependent uptake of rhPRG4, Smad3 phosphorylation and regulation of TGF- β /Smad pathway activation in TGF- β stimulated HEK Blue-TGF- β cells

HEK Blue-TGF- β is an engineered reporter cell line produced by transfecting human embryonic kidney (HEK) cells with TGF- β receptor 1 (TGF- β R1), Smad3 and Smad4 genes (Invivogen, USA). TGF- β 1-treatment results in phosphorylation of Smad3 (pSmad3) and Smad4 (pSmad4), translocation to the nucleus and expression of secreted alkaline phosphatase (SEAP). The activity of SEAP can be detected colorimetrically in media supernatants using a specific substrate (Quanti Blue) at 620-655 nm (Invivogen). HEK-TGF- β cells (500,000) were plated onto sterile chamber slides and incubated with rhodamine-rhPRG4 (25 μ g/mL) for 1 hour $\pm$ anti CD44 or IC antibodies (2 μ g/mL for both antibodies). Cells were pre-incubated with the antibodies for 1-hour prior to rhPRG4 addition. Subsequently, cells were washed with PBS and intracellular CTCF was determined as described above. In another set of experiments, rhodamine labeled rhPRG4 (25 μ g/mL) was incubated with HEK-TGF- β cells and CD44 was probed using FITC-conjugated anti-CD44 antibody (Abcam) and CD44 [198] and rhPRG4 (red) co-localization was examined using a confocal microscope.

Immunostaining of pSmad3 was performed using an anti-Smad3 antibody that detects phosphorylated serine residues (1:1,000 dilution overnight at 4°C; Abcam). HEK-TGF- β cells were stimulated with TGF- β 1 $\pm$ rhPRG4 (150 μ g/mL) for 1 hour. Subsequently, cells were fixed,

washed with PBS and permeabilized as described above. Following probing with anti-pSmad3 antibody, cells were washed with PBS and incubated with goat anti-rabbit IgG (Alexa Fluor 488) (1:1,000 dilution for 1 hour at room temperature; Abcam). Cells were subsequently washed with PBS and green immunofluorescence was quantified across all experimental groups. To investigate the impact of rhPRG4 on TGF- β /Smad pathway activation, HEK-TGF- β cells (100,000 cells/well) were cultured in sterile 96 well plates $\pm$ TGF- β 1 (1ng/mL) $\pm$ rhPRG4 (50, 100 or 150 μ g/mL) for 24 hours in Quanti-Blue media and the 655 nm absorbance intensity was determined. Data are presented as fold absorbance intensities across experimental groups normalized to untreated controls.

4.2.3 TGF- β induction of stress fibers, focal adhesions (FAs) and cell migration in murine NIH3T3 fibroblasts, comparison of stress fibers and FAs in Prg4^{+/+} and Prg4^{-/-} synovial fibroblasts and role of rhPRG4 in regulating fibroblast migration

Murine fibroblasts (NIH3T3; ATCC; 20,000 cells/well) were cultured on collagen type I-coated 22-mm glass coverslips for 24 hours in DMEM supplemented with 10% bovine calf serum (BCS). NIH3T3 cells were starved in DMEM containing 1% BCS for 24 hours. Murine fibroblasts were treated with murine TGF- β 1 (1ng/ml) $\pm$ rhPRG4 (200 μ g/mL) for 24 hours in serum-free DMEM. Subsequently, fibroblasts were fixed using neutral buffered formalin, washed with PBS, permeabilized using 0.1% triton X-100 and probed using an anti- α -SMA (marker of stress fibers; 1:1,000) or anti-vinculin (marker of FA complex; 1:1,000) overnight at 4°C. After washing with PBS, cells were incubated with goat anti rabbit IgG (Alexa Fluor[®]488) at 1:1,000 dilution for 1 hour at 4°C. Rhodamine-labeled phalloidin (cytoskeleton label; 1:1,000) was added with the secondary antibody. All antibodies were obtained from Abcam. Cells were washed and mounted with DAPI medium for 2 hours and viewed under a confocal microscope. The number of stress fiber-positive fibroblasts was determined by a blinded investigator. At least 100 cells over at least

5 different fields were evaluated, and the percentage of stress-fiber positive fibroblasts was calculated. Separately, mean fibroblast cell spread area was determined using NIS-Elements imaging software (Nikon). The number and mean size of FAs per cell were determined using ImageJ software as previously described [222]. Fibroblast migration was determined using a scratch assay. A 1,000 μ L pipette tip was used to perform a uniform scratch in the confluent NIH3T3 fibroblast monolayer. TGF- β (1ng/mL) stimulation was performed for 48 hours $\pm$ rhPRG4 (200 μ g/mL). Subsequently, cells were washed and stained (Cell Biolabs) and imaged using an all-in-one fluorescence microscope (Keyence). A region of interest was defined and scratch widths were determined across multiple locations. Cell migration was expressed as percent wound closure across different experimental groups as previously described [223].

The phenotype of *Prg4*^{-/-} mice is characterized by synovial membrane hyperplasia, chondrocyte apoptosis, and cartilage surface fibrillation [180]. The *Prg4*^{-/-} mouse colony is maintained by Dr. Gregory Jay at Rhode Island Hospital (RIH) and is commercially available (stock no. 025737; JAX, USA). All animal experiments were approved by the IACUC committee at RIH and all experiments were performed according to all applicable guidelines and regulations. Synoviocyte isolation from *Prg4*^{-/-} and *Prg4*^{+/+} synovial tissues was performed as previously described [154, 220]. *Prg4*^{-/-} and *Prg4*^{+/+} synoviocytes were plated onto sterile chamber slides (Thermo Fisher Scientific) and allowed to adhere for 48 hours. Subsequently, cells were stained for α -SMA and vinculin as described above. The mean size of FAs per cell in *Prg4*^{-/-} and *Prg4*^{+/+} synoviocytes was determined as described above. *Prg4*^{-/-} and *Prg4*^{+/+} synoviocytes were seeded in 6 well plates and a scratch was performed in the confluent cell monolayer as described above. Basal synovial fibroblast migration of both genotypes was quantified over 48 hours $\pm$ rhPRG4 (200 μ g/mL), anti-CD44 (2 μ g/mL) or IC (2 μ g/mL) antibodies as previously described [223].

4.2.4 Generation of Active TGF- β in lipopolysaccharide-stimulated murine macrophage J774A and NIH3T3 fibroblast co-culture and impact of rhPRG4 treatment on fibroblast migration

Murine J774A macrophages (ATCC) were cultured in DMEM medium + 10% FBS. Macrophages (300,000 cells in DMEM medium + 10% FBS) were stimulated with lipopolysaccharide (LPS; Invivogen) (5 μ g/mL) for 24 hours. Subsequently, murine macrophages were washed five times with DMEM medium and transferred to the top chamber of a transwell co-culture system (0.4 μ m pore size; Sigma Aldrich). Murine NIH3T3 cells were seeded in the lower chamber of the transwell system and a scratch was performed as described above. NIH3T3 migration was determined following a 48-hour incubation of fibroblasts with macrophages in the co-culture system as described above $\pm$ rhPRG4 (200 μ g/mL). Active and total TGF- β media levels were determined using an ELISA (R&D Systems).

4.2.5 Age-dependent expression of fibrotic markers in synovial tissues from *Prg4*^{GT/GT} animals and the role of PRG4/CD44 interaction in modulating synovial fibrosis in vivo

The *Prg4* gene-trap (*Prg4*^{GT}) mouse colony is maintained by Dr. Gregory Jay at RIH and is commercially available (stock no. 025740; JAX) [224]. *Prg4*^{GT} animal is a genetically engineered PRG4-deficient mouse where the *Prg4* expression can be restored via CRE-mediated recombination [224]. The *Prg4*^{GT/GT} mouse recapitulates the hallmark findings in *Prg4*^{-/-} mouse, namely synovial tissue hyperplasia and cartilage surface fibrillations and recombination in 3-weeks old animals improved but did not completely normalize joint pathological findings [224]. In our studies, recombination (*Prg4*^{GTR/GTR}) occurred in 3-weeks old animals via intraperitoneal injection of tamoxifen (0.1 mg/g in 100 μ L corn oil vehicle) daily for 10 days. We compared gene expression and immunostaining of fibrotic markers: α -SMA, COL1A1 (collagen type-I) and PLOD2 in 2-months old *Prg4*^{GT/GT}, 2-months old *Prg4*^{GTR/GTR}, 9-months old *Prg4*^{GT/GT} and 9-

months old *Prg4*^{GTR/GTR} animals. *ACTA2*, *COL1A1* and *PLOD2* expression levels in murine synovial tissues were performed as previously described [220]. Tissues from each three consecutive mice were pooled and underwent RNA isolation, generating five pooled samples in each experimental group. Separately, animal joints (n=5 in each group) underwent decalcification and paraffin-embedded sectioning as previously described [224]. Immunostaining of synovial tissues was performed using primary antibodies against α -SMA, COL1A1 or PLOD2 (1:1,000 dilutions performed overnight at 4°C) (All antibodies were purchased from Abcam). Subsequently, sections were washed and incubated with goat anti-rabbit IgG (Alexa Fluor 488) (1:1,000 dilution) for 1 hour and fluorescence intensities (expressed as lumens/mm²) were quantified using a fluorescence microscope.

To appreciate the significance of PRG4 and CD44 interaction in the context of fibrotic markers' expression in the synovium, *Prg4*^{GT/GT} animals were crossed with *Cd44*^{-/-} mice (stock no. 005085; JAX) [135] to generate *Cd44*^{-/-}&*Prg4*^{GT/GT} animals. Recombination occurred in 3-weeks old animals to generate *Cd44*^{-/-}&*Prg4*^{GTR/GTR} animals as described in the last paragraph. Histological analyses of joints harvested from 2-months old animals (at least 4 animals per group) were performed as described above, using *Cd44*^{+/+}&*Prg4*^{GT/GT} animals from the same litters as controls. We probed synovial tissues for the following fibrotic markers: α -SMA and PLOD2 using specific primary antibodies (1:1,000 dilutions) followed by goat anti-rabbit IgG (Alexa Fluor 488) and quantitation of fluorescence intensities (expressed as lumens/mm²) as described above.

4.3 Statistical Analyses

Target gene expression was statistically evaluated by comparing ΔC_t (C_t value of target gene- C_t value of GAPDH in the same sample) values of different experimental groups. Statistical significance comparing two groups or multiple groups with parametric data was assessed by Student's *t* test or ANOVA followed by *post-hoc* multiple comparisons (Tukey's *post-hoc* test). Statistical significance comparing two groups or multiple groups with nonparametric data was assessed by Rank Sum test or ANOVA on the ranks. A *p* value of < 0.05 was considered statistically significant. Data are presented as scatter plots with mean and standard deviations highlighted.

4.4 RESULTS

4.4.1 PRG4 reduced ACTA2 expression and stress fiber formation in osteoarthritic fibroblast-like synoviocytes (OA FLS) and CD44 was involved in the uptake of rhPRG4 by OA FLS, whereas rhPRG4-CD44 interaction affected ACTA2 expression in response to TGF- β

PRG4 and HA demonstrated equivalent efficacy in reducing *ACTA2* expression in OA FLS (Fig. 4.1A). Both treatments reduced *ACTA2* expression compared to the positive control TGF- β 1 ($p < 0.001$ for TGF- β 1 + PRG4 or TGF- β 1 + HA against TGF- β 1 alone). In addition to reducing *ACTA2* expression, PRG4 reduced α -SMA content in OA FLS ($p < 0.001$ versus TGF- β 1 alone; Fig. 4.1C). A similar effect was also observed for HA treatment ($p < 0.001$ versus TGF- β 1 alone; Fig. 4.1C). The effect of PRG4 was biologically significant with approximately 52% reduction in mean α -SMA immunofluorescence compared to TGF- β 1 alone. In addition to reducing α -SMA in OA FLS, PRG4 and HA treatments prevented the formation of stress fibers in OA FLS in response to TGF- β 1 stimulation (Fig. 4.1B). rhPRG4 was rapidly internalized by OA FLS and the mechanism of its internalization was related to its interaction with CD44. This was illustrated by

a reduction in OA FLS CTCF with an anti-CD44 treatment (Fig. 4.1D & Fig. 4.1E). Neutralization of CD44 receptor did not completely block rhPRG4 uptake by OA FLS (Fig. 4.1E). Alternatively, treatment with an IC antibody did not alter rhPRG4's uptake by OA FLS ($p>0.05$; Fig. 4.1E). Inhibition of *ACTA2* expression by rhPRG4 was reversed by pre-incubation with an anti-CD44 antibody ($p<0.05$ for TGF- β 1 + rhPRG4 + anti-CD44 versus TGF- β 1 + rhPRG4; Fig. 4.1F). In contrast, IC antibody treatment did not alter the magnitude of reduction in *ACTA2* expression observed with rhPRG4 (Fig. 4.1F).

4.4.2 CD44 receptor facilitated rhPRG4 uptake by HEK-TGF β cells and rhPRG4 inhibited TGF- β 1/Smad pathway activation

Representative images demonstrate intracellular red fluorescence following the incubation of rhodamine-labeled rhPRG4 with HEK-TGF β cells (Fig. 4.2A). Antibody-mediated CD44 receptor neutralization reduced the intensity of red fluorescence, indicative of reduced rhPRG4 uptake into HEK-TGF β cells ($p<0.001$ vs. rhPRG4 alone; Fig. 4.2A and Fig. 4.2B). This effect was specific to CD44 receptor, as a non-specific IC antibody pre-incubation did not alter the extent of rhPRG4 uptake into HEK-TGF β cells ($p>0.05$; Fig. 4.2A and Fig. 4.2B). Further evidence of CD44 involvement in rhPRG4 uptake by HEK-TGF β cells is provided by the intracellular co-localization of rhPRG4 (red) and CD44 [198] as shown by arrows (yellow color) (Fig. 4.2A). TGF- β 1 stimulation resulted in Smad3 phosphorylation as shown in representative images (Fig. 4.2C). Intense green fluorescence in TGF- β 1-stimulated HEK-TGF- β cells indicated the formation of pSmad3. rhPRG4 treatment reduced mean pSmad3 staining intensity subsequent to TGF- β 1 stimulation ($p<0.001$; Fig. 4.2D). In addition, rhPRG4 treatment dose-dependently reduced TGF- β 1/Smad pathway activation as shown by a reduction in the formation of SEAP colored product ($p<0.05$ for 100 and 150 μ g/mL concentrations versus TGF- β 1 alone; Fig. 4.2E).

4.4.3 TGF- β induced stress fiber and FAs formation in murine fibroblast NIH3T3 cells resulting in enhanced cell migration, and these effects were reduced with rhPRG4 treatment

Representative images highlight that murine TGF- β induced the formation of stress fibers and vinculin expression and thus FAs formation in NIH3T3 cells (Fig. 4.3A). TGF- β increased the percentage of stress fiber-positive NIH3T3 cells by ~ 5 -folds ($p < 0.001$ against control cells; Fig. 4.3B), and increased mean cell spread area by ~ 2 -folds ($p < 0.001$ against control cells; Fig. 4.3C). rhPRG4 treatment reduced the percentage of stress fiber-positive NIH3T3 cells ($p < 0.001$; Fig. 4.3B) and mean cell spread area ($p < 0.001$; Fig. 4.3C) to control levels. TGF- β increased the number of FAs in NIH3T3 cells by ~ 4 -folds ($p < 0.001$; Fig. 4.3D), and the mean size of FAs per cell by ~ 2 -folds ($p < 0.05$; Fig. 4.3E). Likewise, rhPRG4 treatment reduced the number of FAs ($p < 0.001$; Fig. 4.3D) and mean FA size ($p < 0.05$; Fig. 4.3E) to control levels. A functional outcome of TGF- β induced stress fiber and FAs formation was the enhanced migration of NIH3T3 fibroblasts (Fig. 4.3F). rhPRG4 reduced TGF- β stimulated NIH3T3 fibroblast migration ($p < 0.001$; Fig. 4.3G) while rhPRG4 alone did not change basal NIH3T3 fibroblast migration ($p > 0.05$ against control; Fig. 4.3G).

4.4.4 Lack of Prg4 expression in murine synovial fibroblasts was associated with enhanced stress fiber formation, FA size and enhanced basal cell migration which was reduced by rhPRG4 or anti-CD44 antibody treatments

Representative images demonstrate enhanced α -SMA and vinculin staining in *Prg4*^{-/-} synoviocytes compared to *Prg4*^{+/+} synoviocytes (Fig. 4.4A). The mean size of FAs was ~ 2 -fold higher in *Prg4*^{-/-} synoviocytes compared to *Prg4*^{+/+} synoviocytes ($p < 0.001$; Fig. 4.4B). *Prg4*^{-/-} synoviocytes displayed enhanced basal migration compared to *Prg4*^{+/+} synoviocytes ($p < 0.01$; Fig. 4.4C and Fig. 4.4D). rhPRG4 and anti-CD44 antibody treatments reduced *Prg4*^{-/-} synoviocyte migration ($p < 0.01$ for both treatments; Fig. 4.4D) with no significant difference between both treatments ($p > 0.05$).

4.4.5 LPS stimulation of macrophages increased active and total TGF- β levels in a macrophage and fibroblast co-culture model, induced fibroblast migration and rhPRG4 treatment reduced fibroblast migration without altering active or total TGF- β levels

In response to LPS stimulation and co-culture of macrophages (M) and fibroblasts (F), active TGF- β levels in the macrophage and fibroblast co-culture were higher compared to fibroblasts alone ($p < 0.001$; Fig. 4.5A). Correspondingly, total TGF- β levels in the macrophage and fibroblast co-culture were higher compared to fibroblasts alone ($p < 0.001$; Fig. 4.5A). The increase in active and total TGF- β levels resulted in enhanced fibroblast migration in the macrophage and fibroblast co-culture compared to fibroblasts alone ($p < 0.001$; Fig. 4.5B and Fig. 4.5C). rhPRG4 treatment reduced fibroblast migration in the co-culture model ($p < 0.001$; Fig. 4.5C). The effect of rhPRG4 was not mediated by an alteration in active or total TGF- β levels, shown by the lack of differences in active or total TGF- β concentrations between rhPRG4-treated and untreated cells ($p > 0.05$; Fig. 4.5D).

4.4.6 Lack of *Prg4* expression resulted in progressive synovial tissue fibrosis, shown by enhanced α -SMA, collagen type-I and PLOD2 production and *Prg4* re-expression reduced synovial tissue fibrosis and this effect was evident in CD44-deficient mice

Synovial tissues from *Prg4*^{GT/GT} animals displayed higher *ACTA2*, *COL1A1* and *PLOD2* expression levels compared to *Prg4* wildtype synovia ($p < 0.001$ for all comparisons; Fig. 4.6A, Fig. 4.6D and Fig. 4.6G). Interestingly, lack of *Prg4* expression resulted in a greater increase in *ACTA2* expression compared to *COL1A1* and *PLOD2* ($p < 0.001$ for all comparisons). The expression of fibrotic markers in synovial tissues from *Prg4*^{GT/GT} animals progressed with age. *ACTA2*, *COL1A1* and *PLOD2* expression levels in 9 months-old *Prg4*^{GT/GT} animals were higher than corresponding expression levels in 2 months-old *Prg4*^{GT/GT} animals ($p < 0.001$ for all comparisons). The regulation of fibrotic gene expression in the synovium by PRG4 was further confirmed by the reduction in *ACTA2*, *COL1A1* and *PLOD2* expression in 9 months-old

Prg4^{GTR/GTR} animals compared to age-matched *Prg4*^{GT/GT} animals ($p < 0.001$ for all comparisons). Representative images of synovial tissues stained for α -SMA, collagen type-I and PLOD2 proteins are shown in Figures 4.6B, 4.6E and 4.6H. There was no significant difference in mean collagen type-I staining intensity between 2 months-old *Prg4*^{GT/GT} and age-matched *Prg4*^{GTR/GTR} animals ($p > 0.05$; Fig. 4.6F). Alternatively, mean α -SMA, collagen type-I and PLOD2 staining intensities were lower in 9 months-old *Prg4*^{GTR/GTR} animals compared to age-matched *Prg4*^{GT/GT} animals ($p < 0.05$ for all comparisons; Fig. 4.6C, Fig. 4.6F and Fig. 4.6I).

The contribution of CD44 to PRG4's effect on α -SMA and PLOD2 expression was further studied *in vivo* by comparing histological staining intensities in synovial tissues from *Cd44*^{+/+}&*Prg4*^{GT/GT}, *Cd44*^{-/-}&*Prg4*^{GT/GT}, and *Cd44*^{-/-}&*Prg4*^{GTR/GTR} (Fig. 4.7). While mean α -SMA staining intensity trended higher in *Cd44*^{-/-}&*Prg4*^{GT/GT} synovia compared to *Cd44*^{+/+}&*Prg4*^{GT/GT} synovia, this increase did not reach statistical significance ($p = 0.16$). Mean α -SMA staining intensity in *Cd44*^{-/-}&*Prg4*^{GTR/GTR} synovia was lower than corresponding mean intensity in *Cd44*^{-/-}&*Prg4*^{GT/GT} synovia ($p < 0.05$). The absence of CD44 receptor increased PLOD2 staining in synovial tissues as evidenced by a higher mean staining intensity in *Cd44*^{-/-}&*Prg4*^{GT/GT} animals compared to *Cd44*^{+/+}&*Prg4*^{GT/GT} animals ($p < 0.001$). In addition, *Prg4* re-expression reduced mean PLOD2 staining in otherwise *Cd44* null animals ($p < 0.001$).

4.5 DISCUSSION

PRG4, also called lubricin and superficial zone protein, is a mucinous glycoprotein with a protein core of 1,404 amino acids and a central mucin domain that is extensively glycosylated with O-linked β (1-3) Gal-GalNAc oligosaccharides [195, 217, 225, 226]. PRG4 is secreted from synovial fibroblasts and superficial zone chondrocytes and is found adsorbed on articular surfaces and the

synovial membrane in addition to its presence in SF [168, 227]. PRG4 has a multifaceted role in the joint and one aspect of its role is to provide boundary lubrication, to prevent friction-induced mitochondrial dysregulation and chondrocyte apoptosis [228-230]. Synthesis of PRG4 by cartilage and synovium was reduced in posttraumatic OA (PTOA) animal models and inflammatory cytokines *e.g.* IL-1 β reduced PRG4 secretion by synovial fibroblasts while TGF- β produced an opposite effect [184, 231, 232]. Furthermore, SF PRG4 levels decreased following acute joint trauma and in patients with advanced OA and rheumatoid arthritis (RA) [233-235]. Therapeutically, native and rhPRG4 demonstrated disease-modifying activities in pre-clinical PTOA mediated by their ability to reduce cartilage degeneration, enhance cartilage repair and reduce chondrocyte apoptosis [179, 236-238].

In this investigation, we studied the role of CD44 in mediating rhPRG4's antifibrotic and antimigratory biological effects on fibroblasts in response to exogenously introduced recombinant TGF- β or TGF- β produced by co-cultured macrophages in response to TLR4 receptor stimulation. We identified that PRG4 and HA provide a similar outcome in TGF- β stimulated OA FLS in the form of preventing transition of fibroblasts to myofibroblasts with inhibition of α -SMA expression and thus stress fiber formation. PRG4 and HA produced an antifibrotic effect in OA FLS using concentrations that are well below what is normally found in SF [233-235]. As HA is the prototypical CD44 ligand, we used HA as a positive control and our findings support that CD44 modulates fibroblast to myofibroblast transition in OA synoviocytes. PRG4 was rapidly internalized by OA FLS, with a prominent role for CD44 in this process. However, CD44 was not the only mechanism by which OA synoviocytes internalized rhPRG4 as approximately 60% of rhPRG4 entry was blocked subsequent to CD44 receptor neutralization. CD44-mediated rhPRG4's uptake partially contributed to the latter's antifibrotic role as rhPRG4's effect on α -SMA

expression was significantly weakened, but not completely abolished, when CD44 mediated uptake was blocked. This indicates that CD44 engagement by rhPRG4 is biologically significant in the context of rhPRG4's antifibrotic effect but does not completely account for the manner in which rhPRG4 interacts with synoviocytes. CD44 also played a role in facilitating the enhanced migration of murine synovial fibroblasts that lack *Prg4* expression as antibody-mediated neutralization of the receptor reduced cell migration. This finding is consistent with our prior observation that *Prg4* null synoviocytes had higher CD44 receptor expression levels compared to *Prg4* competent synoviocytes and is supported by an independent finding that CD44 plays a role in facilitating cell migration [127, 154, 236]. Synovial fibrosis developed in *Prg4* null mice and expression of SMA, PLOD2 and collagen type-I progressively increased as animals aged. Interestingly, the *in vivo* antifibrotic effect due to *Prg4* re-expression was not dependent on CD44 as endogenously produced PRG4 reduced SMA and PLOD2 expression in *Cd44* null animals.

CD44 is a single-pass transmembrane glycoprotein that is widely expressed in immune and connective tissues, and different isoforms, generated by alternate splicing, have been characterized [127, 236]. The extracellular domain of CD44 binds different ligands and thus can sense a broad array of microenvironmental signals resulting in ligand-specific effects in inflammation, cell migration and growth [239-241]. In addition to HA and PRG4, other CD44 ligands include osteopontin, chondroitin, fibronectin, and matrix metalloproteinases (MMPs) [239, 242]. The intracellular domain of CD44 lacks intrinsic kinase activity, but it transduces extracellular ligand-specific signals via a combination of proteolytic fragmentation or interactions with different signaling pathways including members of *Src* and Ras family of GTPases and protein phosphatase-2A [239, 243, 244]. HA is cellularly uptaken by CD44, and this interaction mediates HA's ability to suppress MMP-13 and aggrecanase-1 expression in OA chondrocytes and synoviocytes [128,

245, 246]. In addition to inhibiting synovitis, HA treatment inhibited synovial fibrosis in a murine model of TGF- β and treadmill running [128, 247, 248]. The effect of HA was CD44-dependent as its antifibrotic effect was abolished in CD44-deficient mice [247]. Contrary to HA, PRG4 had an *in vivo* antifibrotic effect that was not dependent on CD44. This might be related to PRG4's ability to regulate other signaling pathways apart from CD44. PRG4 binds to and inhibits the activation of TLR2 and TLR4 receptors by their ligands and by SF aspirates from patients with OA [249, 250]. In addition, PRG4 was internalized by murine macrophages and co-localized with both CD44 and to lesser extent TLR2 receptors in the cytosol of these cells [251]. As such, it is expected that the enhanced TLR2 and TLR4 receptor signaling in the absence of PRG4 would propagate synovitis in *Prg4* null mice. The innate immune-mediated synovitis would in turn contribute to the development of synovial fibrosis. Upon *Prg4* re-expression in 3-weeks old animals, PRG4 would attenuate TLR2 and TLR4 mediated synovitis, thereby potentially reducing TLR-regulated fibrotic tissue remodeling [252].

Myofibroblasts are characterized by α -SMA expression, stress fiber and FAs formation and are thought to play an effector role in the pathophysiology of tissue fibrosis [252, 253]. Compared to fibroblasts, myofibroblasts produce more collagen; respond to mitogenic signals resulting in cell proliferation, migration and secretion of matrix-degrading enzymes [190, 252]. While stress fibers and FAs are unique structures within the cell, they are highly interconnected, where they coordinate cell responses to mechanical signals in their microenvironment [254]. Interestingly, the mean size of FAs, and not their molecular composition or density, can accurately predict cell migration [255]. Normal synovial fibroblasts do not appear to express α -SMA, even with TGF- β stimulation [191]. In our study, OA FLS had a basal level of α -SMA expression and in response to TGF- β , α -SMA expression was enhanced and stress fibers appeared. This was also evident in

murine NIH3T3 fibroblasts as the transition from a fibroblast to a myofibroblast phenotype was accompanied by stress fiber and FAs formation, with subcellular and cellular changes in the form of increased number and mean size of FAs and cell spread area and thus enhanced cell migration. rhPRG4 acted biologically to reduce the formation of stress fibers and to reduce the mean number and size of FAs in NIH3T3 fibroblasts and this reduction resulted in inhibition of fibroblast migration. Thus, it is unlikely that the observed antimigratory effect of rhPRG4 was a physical effect consequent to PRG4's amphipathic nature and its adsorption on hydrophilic and hydrophobic surfaces [198]. The higher mean size of FAs in synoviocytes from *Prp4* null mice, and its resultant enhanced migration argues that PRG4 plays a role in regulating the expression of the cellular machinery mediating migration *in vivo*. This may have a biological relevance in the realm of synovial fibrosis where PRG4 may function to reduce the migration of fibrocytes to the inflamed joint and therefore the likelihood of adding to the pool of existing myofibroblasts in the synovium [256, 257].

Synovial tissue macrophages are critical regulators of the initiation, maintenance and resolution phases of synovitis [258]. The complex functions of macrophages are enabled by the presence of different macrophage subpopulations within the joint, which fulfill distinct inflammatory and antiinflammatory roles [258]. In synovial tissue fibrosis, bone marrow derived monocytes and macrophages are found in increased numbers, which may signal their involvement in the development of synovial fibrosis [259, 260]. In response to an inflammatory signal, macrophages produce TGF- β as a regulatory feedback signal to aid in the resolution of inflammation [261, 262]. TGF- β in turn triggers myofibroblast differentiation and production of matrix proteins [220]. In our study, LPS stimulation resulted in increased secretion of TGF- β by macrophages. Cells secrete a latent form of TGF- β , which in turn is proteolytically converted to active TGF- β by a variety of

proteases, including MMPs [263]. The generation of active TGF- β in the co-culture system was associated with fibroblast migration. Interestingly, the introduction of rhPRG4 after macrophage activation did not alter total TGF- β quantity produced by macrophages. Furthermore, rhPRG4 did not alter the rate of activation of latent TGF- β , indicating that the antimigratory effect of rhPRG4 was at the level of fibroblasts' response to TGF- β receptor activation. This is supported by our observation that rhPRG4 reduced Smad3 phosphorylation in TGF- β reporter cells and that reduction in pSmad3 intracellular levels attenuated the TGF- β /Smad pathway activation. In TGF- β reporter cells, the attenuation of Smad signaling pathway was downstream to rhPRG4 uptake by these cells in a mechanism that involved CD44 receptor, similar to what was observed in OA FLS. However, the precise mechanism that is activated by CD44 engagement that inhibits TGF- β /Smad signaling pathway remains unclear. Another limitation of our study was that we did not study the antifibrotic effect of rhPRG4 *in vivo*.

4.6 CONCLUSION

In summary, we demonstrated that PRG4 is an important regulator of synovial tissue fibrosis using a combination of *in vitro* and *in vivo* models. PRG4 prevented the transition of human and murine fibroblasts to a myofibroblast phenotype and in OA FLS; the mechanism of PRG4's antifibrotic effect was partially linked to CD44-mediated cell uptake. rhPRG4 acted biologically to reduce Smad3 phosphorylation, the activation of TGF- β /Smad signaling pathway, and FA mean size which translated to inhibiting fibroblast migration. The lack of *Prg4* expression in murine synovial tissues resulted in enhanced expression of SMA, collagen type-I and PLOD2, the enzyme responsible for collagen cross-linking. Synovial fibrosis in *Prg4*^{GT/GT} animals progressed with age and re-establishing *Prg4* expression was antifibrotic. The role of PRG4 in regulating synovial fibrosis *in vivo* extends beyond its interaction with CD44 receptor, as *Prg4* re-expression was

antifibrotic in *Cd44* null mice. Therapeutically, overexpressing PRG4 in the articular joint, using a viral mediated gene delivery approach, may prove beneficial in reducing synovial fibrosis in addition to reducing cartilage degeneration [264, 265]. The study of PRG4 as an antifibrotic modulator of the joint's soft tissues is further warranted especially with the known contribution of synovial fibrosis in advanced OA.

4.7 DECLARATIONS

4.8 Ethical Approval and Consent to Participate

-OA synoviocytes were obtained from a commercial source (Cell Applications Inc.). Harvest of synoviocytes was performed following appropriate IRB approvals from partner sites with informed written consent from the donor. Patients were de-identified. Authors did not seek patients' consent to study these biological samples. This study was approved by the IRB at Chapman University, Orange, CA, USA.

-Harvest of synovial tissues from mice, and histological evaluations of mice joints were approved by the IACUC Committee at Rhode Island Hospital, Providence, RI, USA. All experiments were performed in accordance with established guidelines and regulations.

4.9 Consent for Publication

Not applicable

4.10 Availability of Supporting Data

Not applicable

4.11 Competing Interests

Authors MQ, HR and LZ have nothing to disclose.

Author GJ authored patents on rhPRG4 and holds equity in Lubris LLC, MA, USA.

Author TS authored patents on rhPRG4, is a paid consultant for Lubris LLC, MA, USA and holds equity in Lubris LLC, MA, USA.

Author KE authored patents on rhPRG4.

All authors have no non-financial competing interests related to this manuscript.

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4.13 Authors' Contributions

Authors MQ, GJ, LZ, and KE carried out experiments and participated in analysis of data. Authors TS and HR participated in study design and critical interpretation of results. Authors GJ and KE conceived the study and participated in data analysis and interpretation. All authors have participated in drafting and critical evaluation of the manuscript. All authors have read and approved the final version of the manuscript.

4.14 Acknowledgements

Not applicable

4.15 Figures

Fig. 4.1 Impact of proteoglycan-4 (PRG4) and high molecular weight hyaluronic acid (HA) treatments on TGF-β1 induced alpha smooth muscle actin (α -SMA) expression (*ACTA2*), production and stress fiber formation in fibroblast-like synoviocytes isolated from patients with OA (OA FLS) undergoing knee replacement surgery and CD44-dependent uptake of rhodamine-labeled recombinant human PRG4 (rhPRG4) into OA FLS and associated regulation of *ACTA2* expression. OA FLS were treated with TGF-β1 (1 ng/mL) $\pm$ rhPRG4 (~240 kDa) or HA (~1,200 kDa) (100 μ g/mL for both treatments) for 24 hours followed by RNA isolation and qPCR using *GAPDH* as an internal reference gene. OA FLS were stained with anti- α -SMA [198] and counterstained for α -tubulin (red) and DAPI (blue). Corrected total cell fluorescence (CTCF) of α -SMA was determined following a 48-hour treatment, and normalized to controls. Rhodamine-labeled rhPRG4 was incubated with OA FLS $\pm$ anti-CD44 or isotype control (IC) antibodies for

30 min., and CTFC was quantified. OA FLS were treated with TGF- β 1 $\pm$ rhPRG4 $\pm$ anti-CD44 or IC for 24 hours and *ACTA2* expression was determined. Data are presented as a scatterplot with means and standard deviations highlighted utilizing OA FLS from 3-4 different patients. * $p < 0.001$; *** $p < 0.05$. Scale=50 μ m.

A) PRG4 and HA reduced *ACTA2* expression in OA FLS.

B) Representative images showing TGF- β 1 induced stress fiber formation in OA FLS (white arrows) and PRG4 or HA treatments prevented their formation.

C) PRG4 and HA reduced α -SMA CTCF in OA FLS.

D) Representative images showing rhodamine-rhPRG4 intracellular localization (white arrows), following incubation with OA FLS.

E) Co-incubation of rhPRG4 and anti-CD44 reduced cellular uptake of rhPRG4.

F) Anti-CD44 antibody co-treatment reduced rhPRG4's antifibrotic effect in OA FLS.

Fig. 4.2 CD44-dependent interaction of recombinant human proteoglycan-4 (rhPRG4) with HEK Blue-TGF-β cells and associated modulation of phosphorylated Smad3 (pSmad3) and TGF-β/Smad signaling pathway. HEK Blue-TGF-β is an engineered cell line produced by transfecting human embryonic kidney (HEK) cells with TGF-β receptor 1 (TGF-β R1), Smad3 and Smad4 genes. Activation of TGF-β R1 results in expression of secreted alkaline phosphatase (SEAP) whose activity can be determined colorimetrically. Rhodamine-labeled rhPRG4 was incubated with HEK Blue-TGF-β cells ± anti-CD44 or isotype control (IC) antibodies and corrected total cell fluorescence (CTCF) was determined. pSmad3 immunocytostaining was performed using an antibody against pSmad3 and pSmad3 fluorescence intensity was determined. Activity of TGF-β/Smad pathway was determined colorimetrically. * $p < 0.001$; ** $p < 0.01$; n.s.: non-significant. Scale in A=40 μm; Scale in C=50 μm.

- A) Representative images showing rhPRG4 internalization (white arrows) by HEK-TGF-β cells and colocalization with CD44 receptor (white arrows in merged image).
- B) rhPRG4 uptake by HEK-TGF-β cells was reduced by CD44 receptor neutralization.
- C) Representative images showing pSmad3 staining in HEK-TGF-β cells following TGF-β stimulation (white arrows) ± rhPRG4 treatment.
- D) pSmad3 immunocytostaining was reduced following rhPRG4 treatment.
- E) rhPRG4 treatment dose-dependently reduced TGF-β/Smad signaling pathway in TGF-β stimulated HEK-TGF-β cells.

Fig. 4.3 Impact of recombinant human proteoglycan-4 (rhPRG4) treatment on TGF-β induced stress fiber formation, vinculin expression, and formation of focal adhesions (FAs) in murine fibroblasts (NIH3T3) and its relationship to cell migration. Stress fiber formation was probed using an anti-alpha smooth muscle actin (α-SMA) antibody and a blinded investigator evaluated % stress fiber-positive fibroblasts. Vinculin (a marker of FAs) expression was probed using an anti-vinculin antibody and cells were counterstained using rhodamine-labeled phalloidin (cytoskeleton label; red). NIH3T3 Fibroblast migration was performed using a scratch assay and the wound closure percentage was determined. * $p < 0.001$; ** $p < 0.01$; *** $p < 0.05$; *n.s.*: non-significant. Scale in A and F=20μm;

- A) Representative images showing TGF-β induced stress fiber formation and vinculin staining (white arrows) in NIH3T3 fibroblasts.
- B) rhPRG4 reduced stress fiber formation in NIH3T3 fibroblasts.
- C) rhPRG4 reduced mean NIH3T3 fibroblast spread area.
- D) rhPRG4 reduced the number of FAs in NIH3T3 fibroblasts.
- E) rhPRG4 reduced the mean size of FAs in NIH3T3 fibroblasts.
- F) Representative images showing NIH3T3 migration in response to TGF-β ± rhPRG4.
- G) rhPRG4 reduced NIH3T3 migration.

Fig. 4.4 Stress fibers and focal adhesions (FAs) in murine synovial fibroblasts isolated from *Prg4*^{-/-} and *Prg4*^{+/+} mice and their relationship to cell migration. Stress fiber formation was probed using an anti-alpha smooth muscle actin (α -SMA) antibody [198]. Vinculin (a marker of FAs) expression was probed using an anti-vinculin antibody [198] and cells were counterstained using rhodamine-labeled phalloidin (cytoskeleton label; red). The impact of rhPRG4 treatment (200 $\mu\text{g/mL}$) on basal *Prg4*^{-/-} synovial fibroblast migration was assessed using the scratch assay and the wound closure percentage was determined. To highlight the CD44-dependency of the enhanced basal migration of *Prg4*^{-/-} synovial fibroblasts, cells were also treated with either an anti-CD44 or isotype control (IC) antibodies (2 $\mu\text{g/mL}$ for both antibodies). * $p < 0.001$; ** $p < 0.01$; n.s.: non-significant. Scale in A=50 μm (α -SMA) and 100 μm (vinculin); Scale in C=50 μm .

- A) Representative images showing increased stress fibers and FAs in *Prg4*^{-/-} synoviocytes.
- B) *Prg4*^{-/-} synoviocytes demonstrated a higher mean FA size than *Prg4*^{+/+} synoviocytes.
- C) Representative images showing enhanced basal migration of *Prg4*^{-/-} synoviocytes compared to *Prg4*^{+/+} synoviocytes.
- D) rhPRG4 and anti-CD44 treatments were equally effective in reducing basal migration of *Prg4*^{-/-} synoviocytes.

Fig. 4.5 Impact of co-culturing murine fibroblasts (F) and murine macrophages (M) on active and total supernatant TGF-β levels, fibroblast migration and efficacy of rhPRG4 in regulating fibroblast migration in the co-culture model. Murine macrophages (J774A) were stimulated with lipopolysaccharide (LPS; 5μg/mL) for 24 hours prior to seeding in the top chamber of a co-culture system. Murine fibroblasts (NIH3T3) were cultured in the lower chamber of the same system. Active and total TGF-β media levels were determined using an ELISA. A scratch was performed in the fibroblast monolayer and fibroblast migration in the lower chamber was determined at 48 hours ± rhPRG4 (200μg/mL). * $p < 0.001$; ** $p < 0.01$; n.s.: non-significant. Scale = 40μm.

- A) Active and total TGF-β concentrations were higher in the fibroblast and macrophage co-culture compared to fibroblasts alone.
- B) Representative images of fibroblast migration across different experimental groups. Fibroblast migration was highest in co-cultured fibroblasts and macrophages and rhPRG4 treatment reduced fibroblast migration.
- C) rhPRG4 reduced fibroblast migration in a fibroblast and macrophage co-culture model.
- D) rhPRG4 did not alter active or total TGF-β media levels in the fibroblast and macrophage co-culture model.

Fig. 4.6 Gene expression and immunohistological analysis of fibrotic markers: alpha smooth muscle actin (α -SMA) (*ACTA2*), collagen type-1 (*COL1A1*) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase (*PLOD2*) in synovial tissues isolated from 2- and 9-months old gene-trap (*Prg4^{GT/GT}*) and 2 and 9 months old recombined gene-trap (*Prg4^{GTR/GTR}*) mice. The *Prg4* gene-trap allele is a loss of function allele (*Prg4^{GT/GT}*) whose function is restored with *Cre*-excision (*Prg4^{GTR/GTR}*). Recombination occurred at 3 weeks of age using intraperitoneal tamoxifen (0.1 mg/g in 100 μ L corn oil vehicle) daily for 10 days. Fibrotic markers were probed using specific antibodies and staining intensities (lumens per mm²) were quantified. * $p<0.001$; ** $p<0.01$; *** $p<0.05$. Scale=100 μ m.

A) *ACTA2* expression was higher in 9 months *Prg4^{GT/GT}* compared to 2 months littermates and was reduced by *Prg4* re-expression.

B) Representative images showing enhanced α -SMA immunostaining in 2 and 9 months *Prg4^{GT/GT}* synovia.

C) *Prg4* re-expression reduced α -SMA content in 2 and 9-months old animals.

D) *COL1A1* expression was higher in 9 months old *Prg4^{GT/GT}* animals compared to 2 months and was reduced by *Prg4* re-expression.

E) Representative images showing enhanced collagen type I immunostaining in 9 months old *Prg4*^{GT/GT} synovia.

F) Collagen type I content was higher in 9 months old *Prg4*^{GT/GT} animals and was reduced with *Prg4* re-expression.

G) *PLOD2* expression was higher in 9 months old *Prg4*^{GT/GT} animals compared to 2 months and was reduced by *Prg4* re-expression.

H) Representative images showing enhanced PLOD2 immunostaining in *Prg4*^{GT/GT} synovia.

I) *Prg4* re-expression reduced PLOD2 content in *Prg4*^{GT/GT} synovia in 2- and 9-months old animals.

Fig. 4.7 Modulation of expression of fibrotic markers: alpha smooth muscle actin (α -SMA) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD2) in synovial tissues from *Prg4* gene-trap animals by CD44. The *Prg4* gene-trap allele is a loss of function allele (*Prg4*^{GT/GT}) whose function is restored with *Cre*-excision (*Prg4*^{GTR/GTR}). *Prg4*^{GT/GT} animals were crossed with *Cd44*^{-/-} mice to generate *Prg4*^{GT/GT}&*Cd44*^{-/-} animals. Recombination occurred at 3 weeks of age using intraperitoneal tamoxifen (0.1 mg/g in 100 μ L corn oil vehicle) daily for 10 days. Fibrotic markers were probed using specific antibodies and staining intensities (lumens per mm²) were quantified. *Prg4* recombination reduced α -SMA and PLOD2 staining in synovial tissues of 2-months old *Cd44*^{+/+} and *Cd44*^{-/-} animals. **p*<0.001; ****p*<0.05; n.s.: non-significant.

5 CHAPTER 5

SUMMARY AND CONCLUSION

In chapter 2, given the importance of CD44 in OA pathogenesis, and the emerging evidence of a biological role for HA, we aimed to evaluate the role of CD44 in regulating NF- κ B activation and proinflammatory cytokine production in response to TLR2 receptor activation in human macrophages, using a combination of CD44 receptor knockdown, TNF- α production by macrophages from CD44 wildtype and knockout mice, and CD44 receptor engagement by CD44-neutralizing Ab and HA treatments. HA is produced by fibroblast-like synoviocytes and is a major component of the synovial fluid (SF). HA exerts important functions in the joint and its biological effects are mediated by its binding to its transmembrane receptor, CD44. In this work, we found that Anti-CD44 Ab and HA treatments reduced NF- κ B translocation, IL-1 β and TNF- α expression, and production. Inhibition of proinflammatory response in macrophages by HA was mediated by CD44. Protein phosphatase 2A mediated the reduction in NF- κ B translocation by HA. CD44 knockdown reduced NF- κ B nuclear translocation and downstream IL-1 β and TNF- α protein production following TLR2 receptor stimulation. *Cd44*^{+/+} murine bone marrow-derived macrophages produced higher TNF- α compared with *Cd44*^{-/-} macrophages following TLR2 stimulation. HA dose-dependently inhibited TLR2-induced TNF- α production by murine bone marrow-derived macrophages. OA SF stimulated TLR2 and TLR4 receptors and induced NF- κ B translocation in human macrophages. Anti-CD44 Ab treatment significantly reduced macrophage activation by OA SF. CD44 regulated TLR2 responses in human macrophages, whereby a

reduction in CD44 levels or engagement of CD44 by its ligand (HA) or a CD44-specific Ab reduced NF- κ B translocation and downstream proinflammatory cytokine production. A CD44-specific Ab reduced macrophage activation by OA SF, and CD44 is a potentially novel target in OA treatment.

In chapter 3, we investigated the antifibrotic efficacy of forskolin, an adenylyl cyclase activator that increases intracellular cAMP, in a model of TGF- β stimulated osteoarthritic fibroblast-like synoviocytes (OA-FLS). Synovial fibrosis is characterized by excess extracellular collagen I, TIMP-1 and PLOD2 expression and FLS proliferation and migration. A myofibroblast-like differentiation of synoviocytes, characterized by alpha smooth muscle actin (α -SMA) expression, is associated with synovial fibrosis. Synoviocytes secrete HA and proteoglycan-4 (PRG4). HA and PRG4 may be linked to synovial fibrosis. Collagen I (COL1A1), α -SMA, TIMP-1, PLOD2 expression and procollagen I, α -SMA, HA and PRG4 production, migration and proliferation of OA-FLS were studied in TGF- β $\pm$ forskolin-treated OA-FLS. The antifibrotic effect of HA and PRG4 was investigated using OA-FLS and *Prg4*^{-/-} synoviocytes, and fibrosis markers were evaluated in *Prg4*^{+/+} and *Prg4*^{-/-} synovial tissues. Forskolin (10 μ M) reduced COL1A1, α -SMA and TIMP-1 expression, with no change in PLOD2 expression. Forskolin (10-30 μ M) reduced procollagen I, α -SMA and OA-FLS migration and proliferation. Forskolin (10 μ M) increased HA secretion, PRG4 expression and production. HA and synoviocyte PRG4 reduced α -SMA expression and content. PRG4 reduced COL1A1 expression and procollagen I content. *Prg4*^{-/-} synovial tissues exhibited increased α -SMA, COL1A1 and TIMP-1 expression compared to *Prg4*^{+/+} tissues. *Prg4*^{-/-} synoviocytes demonstrated strong α -SMA and collagen type I staining with none detected in *Prg4*^{+/+} synoviocytes and PRG4 treatment reduced cellular staining for both proteins. Clinical significance: OA synovial fibrosis can be mitigated by therapeutically increasing

intracellular cAMP in synovial fibroblasts with an associated enhancement of HA and PRG4 production.

In chapter 4, we demonstrated that PRG4 is an important regulator of synovial tissue fibrosis using a combination of *in vitro* and *in vivo* models. PRG4 prevented the transition of human and murine fibroblasts to a myofibroblast phenotype and in OA FLS; the mechanism of PRG4's antifibrotic effect was partially linked to CD44- mediated cell uptake. rhPRG4 acted biologically to reduce Smad3 phosphorylation, the activation of TGF- β / Smad signaling pathway, and FA mean size which translated to inhibiting fibroblast migration. rhPRG4 inhibited fibroblast migration in a macrophage and fibroblast co-culture model without altering active or total TGF- β levels. The lack of *Prg4* expression in murine synovial tissues resulted in enhanced expression of SMA, collagen type-I, and PLOD2, the enzyme responsible for collagen cross-linking. Synovial fibrosis in *Prg4*^{GT/GT} animals progressed with age and re-establishing *Prg4* expression was antifibrotic. The role of PRG4 in regulating synovial fibrosis *in vivo* extends beyond its interaction with CD44 receptor, as *Prg4* re-expression was antifibrotic in *Cd44* null mice. Therapeutically, overexpressing PRG4 in the articular joint, using a viral mediated gene delivery approach, may prove beneficial in reducing synovial fibrosis in addition to reducing cartilage degeneration. The study of PRG4 as an antifibrotic modulator of the joint's soft tissues is further warranted especially with the known contribution of synovial fibrosis in advanced OA.

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