UNIVERSITY OF CALIFORNIA, SAN DIEGO

REGULATION OF ARTICULAR CARTILAGE PROTEOGLYCAN 4 SECRETION IN AGING AND OSTEOARTHRITIS

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Bioengineering

by

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The dissertation of Alexander Y. Hui is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

REGULATION OF ARTICULAR CARTILAGE PROTEOGLYCAN 4 SECRETION IN AGING AND OSTEOARTHRITIS

by

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University of California, San Diego, 2016

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Proteoglycan 4 (PRG4) is a macromolecule synthesized and found within the synovial joint that contributes lubricating and other functions to maintain joint health. PRG4 secretion by articular cartilage is an important parameter of PRG4 mass balance in the synovial joint and is influenced *in vitro* by a number of chemical and mechanical mediators. With joint aging and osteoarthritis (OA), cartilage PRG4

secretion may be altered due to changes in constitutive and induced chondrocyte expression and changes in SF regulation of cartilage. The overall motivation of this dissertation was to contribute to an understanding of the changes in regulation of articular cartilage PRG4 secretion by TGF-β1, a potent stimulus, and by human synovial fluid (hSF), a complex mixture that interacts with cartilage physiologically, with aging and OA.

In normal human articular cartilage, constitutive and TGF-β1-stimulated PRG4 production decreased with increasing age, and these age-dependent declines were associated with decreases with age in the number of PRG4+ chondrocytes without a marked alteration in the density of chondrocytes in the superficial zone. Changes in activity and responsiveness of chondrocytes likely underlie age-related declines in PRG4 production. Age-associated decrease in cartilage constitutive and TGF-β1-stimulated PRG4 production may be related to the age-associated predisposition to cartilage degeneration and OA. hSF contains endogenous regulators of cartilage PRG4 secretion, resulting in a net stimulation. Cartilage PRG4 secretion was substantially stimulated by hSF from OA patients (OA-hSF), compared to hSF from normal cadaveric donors (NL-hSF) and pooled human serum (HS) from normal adult donors. OA-hSF stimulation of cartilage PRG4 secretion was not substantially modulated by inhibition of the TGF-β, IL-1, and/or TNF signaling pathways, individually.

Elucidating the age- and OA- related changes in cartilage PRG4 secretion, due to alterations in constitutive and induced chondrocyte expression and alterations in hSF regulation of cartilage, is one step towards a systems-based understanding of synovial joint PRG4 homeostasis and derangement in health, aging, and disease.

CHAPTER 1:

INTRODUCTION

1.1 General Introduction to the Dissertation

Proteoglycan 4 (PRG4) is a macromolecule synthesized and found within the synovial joint that contributes lubricating and other functions to maintain joint health. PRG4 secretion by cartilage is an important parameter of PRG4 mass balance in the synovial joint. Physiologically, synovial fluid (SF) interacts with cartilage and provides metabolic, mechanical, and regulatory functions. With joint aging and osteoarthritis (OA), cartilage PRG4 secretion may be altered due to changes in constitutive and induced chondrocyte expression and changes in SF regulation of cartilage.

The overall motivation of this dissertation was to contribute to an understanding of the changes in regulation of articular cartilage PRG4 secretion by TGF-β1, a potent stimulus, and by human SF (hSF), a complex mixture that interacts with cartilage physiologically, with aging and OA. Specifically, the objectives of this work were to: (1) characterize, in normal human articular cartilage of the lateral femoral condyle, the relationship with donor age of cartilage PRG4 production, constitutively and with TGF-β1 stimulation, and to determine if any age-related changes are due to changes in cell responsiveness or cell number (**Chapter 2**), (2) determine if SF from normal joints of human cadaveric knees (NL-hSF) and SF from

patients undergoing total knee arthroplasty (OA-hSF) regulate cartilage PRG4 secretion, and if regulation is mediated by endogenous regulators in SF activating the TGF-β, IL-1, and TNF signaling pathways in cartilage (**Chapter 3**), and (3) determine if LY2109761, a TGF-β receptor type I and II kinase dual inhibitor, inhibits OA-hSF-stimulated cartilage PRG4 secretion (**Chapter 4**).

Chapter 1 begins with a review of the synovial joint system, its components and interactions, in health, and their changes with aging and disease. This is followed by background on SF and articular cartilage. PRG4 function and structure is then discussed. Finally, changes in the synovial joint system with disease are reviewed. Portions of Chapter 1 are reproduced from *Wiley Interdiscip Rev Syst Biol Med*, 4(1), Hui AY, McCarty WJ, Masuda K, Firestein GS, Sah RL, A systems biology approach to synovial joint lubrication in health, injury, and disease, p. 15-37, Copyright (2011), with permission from John Wiley & Sons, Inc.

Chapter 2, which has been submitted to the journal *Arthritis and Rheumatology*, assessed the relationship with age of PRG4 production by human knee articular cartilage, constitutively, and in response to TGF-β1, and investigated possible explanations for these age-related observations. Aging was associated with diminished PRG4 secretion by normal human femoral condyle cartilage, predominantly due to a marked reduction in the number of PRG4+ chondrocytes, but not overall cellularity. The age-associated decrease in cartilage constitutive and TGF-β1-stimulated PRG4 production may be related to the age-associated predisposition to cartilage degeneration and OA.

Chapter 3 studied cartilage PRG4 secretion response to NL-hSF and OA-hSF as well as evaluated the role of endogenous regulators in OA-hSF for several of the most potent *in vitro* regulators of PRG4 secretion. hSF contained endogenous

regulators of cartilage PRG4 secretion, causing a net stimulation, with OA-hSF demonstrating higher potency compared to NL-hSF and HS. OA-hSF stimulation of cartilage PRG4 secretion was not modulated by inhibition of TGF-β, IL-1, nor TNF signaling pathways, individually.

Chapter 4 studied cartilage PRG4 secretion in response to OA-hSF and LY2109761, a blocker of TGF-β1 activity. Unexpectedly, there was a significant interaction effect between LY2109761 and OA-hSF, markedly stimulating cartilage PRG4 secretion.

Chapter 5 discusses the major findings and future directions for this work.

1.2 Synovial Joint Overview

Synovial joints are the most common joints of the human body and allow load-bearing, low-friction, wear-resistant movement between apposing bone surfaces [7]. Synovial joints normally include a cavity filled with SF. The SF cavity is surrounded by, articular cartilage covering the bone surfaces, and a fibrous capsule including the synovium, the inner lining.

SF has biomechanical, metabolic, and regulatory functions. SF is normally a clear, straw-colored, viscous liquid. In normal human knee joints, the volume of SF is ~1 ml [89]. One main function of SF is to lubricate cartilage in synovial joints, facilitating low-friction and low-wear articulation [7]. The molecular and cellular constituents within SF give rise to its unique properties and functions in maintaining joint homeostasis. SF is composed of a blood plasma dialysate and molecules secreted by cells lining and within the synovial joint space, including the lubricant molecules, hyaluronan (HA) and PRG4 (also known as lubricin and superficial zone protein) [44, 75, 96]. Additionally, SF serves metabolic functions, facilitating the transport of

nutrients, waste products, and other metabolites to and from synovial tissues, as well as enzymes that act upon and within these tissues. Finally, SF contains soluble molecules, such as morphogens, growth factors, and cytokines, which mediate communication between cell populations in the joint [10, 35, 42, 74].

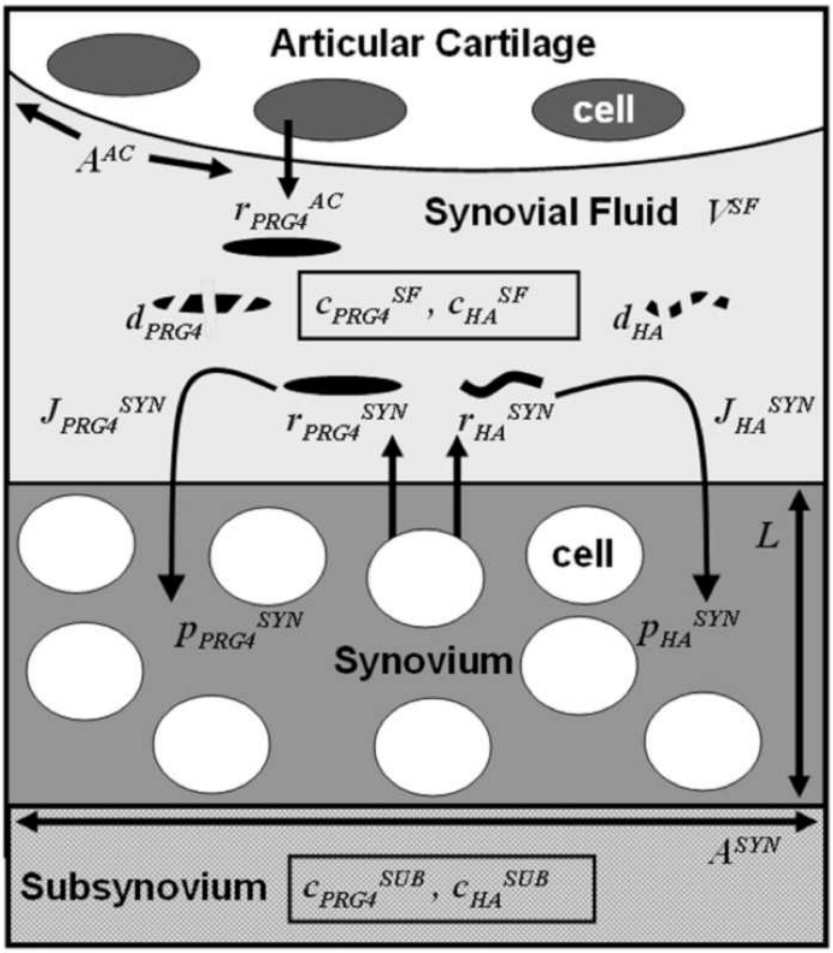
Joint disease and injury can result in pain and dysfunction of synovial joints. OA is a degenerative disease that results in destructive changes to joint structures including cartilage, synovium, and bone. OA has been classified as idiopathic or secondary to other factors, such as joint trauma and congenital diseases. Knee OA has been classified according to clinical, laboratory, and radiographic criteria [1]. Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by joint swelling, joint tenderness, and destruction of synovial joints [77]. Synovitis is a key clinical feature of RA, and together with other criteria including the number of joints involved, duration of symptoms, and test results for serology and acute-phase reactants, can be used to classify RA [77, 113]. Traumatic joint injury can result in cartilage damage, articular and bone fractures, damage to soft tissues such as ligaments and menisci, and lesions in the joint capsule and synovium. Such damage is associated with a number of mechanical and biological changes in the synovial joint that contribute to the development of post-traumatic OA [3, 33, 65].

The composition and function of SF are altered in joint injury and disease, both due to changes directly of the SF and changes in tissues of the synovial joint. SF is in direct physical contact with cartilage and synovium, and in some joints, meniscus and ligament. SF interacts with and mediates interactions between synovial joint tissues. These tissues may themselves be altered in injury and disease. Changes in cellular metabolism and structure in these tissues may be reflected by changes in SF composition and function. Such changes in SF may result in a reduced ability to

lubricate articulating cartilage and a catabolic environment within the joint, together contributing to joint deterioration. Alterations in joint tissues may be detrimental, propelling the SF to an aberrant state and leading to joint pathology. Thus, the disease-associated changes observed in SF likely are both exacerbated by and contribute to pathology of the synovial joint.

1.3 SF Composition in Health and Disease

The synovial joint is a complex structure comprising SF bounded by articular cartilage and synovium, which provides semi-permeable retention of solutes. Concentrations of HA and PRG4, and other macromolecules, in the synovial joint are functions of their (1) synthesis by synovial joint resident cells, namely chondrocytes and synoviocytes, (2) loss from the synovial joint by transport and degradation, (3) binding interactions with binding proteins, and (4) SF volume, all of which may be altered in synovial joint states of normal, aging, acute traumatic injury, and OA (**Fig. 1.2**). Changes in these parameters associated with normal aging, OA, and injury may lead to altered SF lubricant composition and function and predispose the joint to further, progressive damage.



Variable or parameter	Units	Description
i		PRG4, HA
c_i^{SF}	mg/ml	Concentration of i in synovial fluid
c_i^{SUB}	mg/ml	Concentration of i in subsynovium
J_i^{SYN}	mg/s	Total flux of i through membrane area
p_i^{SYN}	cm/s	Permeability of i through synovium
r_i^{SYN}	mg/(cm²•s)	Secretion rate of i by synoviocytes (SYN)
r_i^{AC}	mg/(cm²•s)	Secretion rate of i by articular cartilage (AC)
d_i	mg/s	Degradation rate of i in synovial fluid
A^{SYN}	cm²	Area of synovial membrane
A^{AC}	cm²	Area of cartilage surfaces
L	cm	Membrane thickness
V^{SF}	ml	Volume of synovial fluid

Figure 1.1: Synovial joint lubricant mass balance. (Figure adapted from [11]).

1.3.1 Plasma Proteins

A major component of SF composition is proteins derived from plasma. Blood plasma and SF share many similarities in their protein composition [90, 91], with a superimposed effect of the sub-synovium and synovium selectively hindering large plasma proteins from entering the synovial joint space from the vasculature. Total protein concentration in normal human SF is 19–28 mg/ml, nearly one-third of that in plasma [40, 60, 89]. Quantitatively, the major protein species in SF is albumin, at a concentration of ~12 mg/ml [60]. Other major protein components of normal SF include β_1, γ, α_1, and α_2 globulins, each at concentrations of 1–3 mg/ml in normal SF [60]. Plasma proteins enter the synovial joint space from the vasculature across a blood-joint barrier that is selectively permeable, largely on the basis of solute size [101], resulting in blood plasma and SF sharing many similarities in their protein composition [90, 91]. Large molecular weight plasma proteins, such as fibrinogen (340 kDa), are at relatively low concentrations in SF; in contrast, small molecular weight plasma proteins such as albumin (69 kDa) and transferrin (90 kDa), are in relatively high concentrations in SF (the concentration of albumin being ~37% of that in plasma) [23, 60, 98].

The protein content and concentration in SF is increased with joint inflammation. Total protein concentration in SF from patients with OA, rheumatoid arthritis RA, and traumatic arthritis is higher than normal [23, 60, 100, 102], indicating structural and functional changes in the synovium with joint injury and disease. Synovial inflammation, as occurs in many joint diseases, compromises the ability of synovium to selectively retain and filter proteins. For example, SF from RA patients has high levels of globulins and glycoproteins, large molecular weight proteins normally not found in normal SF. The distribution of proteins in SF of RA patients is

also altered and more closely resembles that in serum. Large plasma proteins, such as β_2 macroglobulin (1,000kDa), fibrinogen (340kDa), β_1 lipoprotein (3,200kDa), α_2 macroglobulin (820kDa), and α_2 glycoprotein (1,000kDa), are present in SF at increased concentrations and amounts in RA patients [23, 98]. The ratio of blood and SF concentrations of plasma proteins, that are not locally synthesized or degraded within the joint have been used to characterize permeability properties of the blood-joint barrier [60, 101].

1.3.2 Lubricant Molecules

Lubrication of articulating cartilage surfaces by SF is mediated by several lubricant macromolecules synthesized and secreted by synovial cell populations and found in SF. HA [83] and PRG4 [107, 108] are the primary lubricant macromolecules in SF and are present in normal SF at mean concentrations of ~3.2–4.1 mg/ml [22, 37, 103] and ~0.035–0.24 mg/ml [26, 78, 92], respectively. HA is a non-sulfated glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and D-N-acetylglucosamine. HA in normal SF is present as a polydisperse population with a weight average molecular weight of 6–7 MDa, with the majority greater than 4MDa [57]. HA contributes to the viscosity of SF and provides outflow buffering [72]. Products of the *PRG4* gene, which include superficial zone protein (SZP) and lubricin, are mucinous glycoproteins with multiple O-linked β(1-3)Gal-GalNAc oligosaccharides that mediate boundary lubrication of articular cartilage [46]. SZP is a ~345 kDa glycoprotein synthesized and secreted by chondrocytes in the superficial zone of cartilage, and not from other depths of cartilage [96]. Lubricin is a ~220 kDa glycoprotein expressed by synovial fibroblasts and also present in SF [44, 47, 107].

Abnormal joint states are associated with altered concentrations and MW of SF lubricant macromolecules, which may be due to changes in the rates of synthesis,

secretion, degradation, and/or loss from the joint. The mean concentrations of HA in pathological SF are lower than that in normal SF, with SF from OA patients ranging from 1.2–2.2 mg/ml [13, 21, 22, 36] and RA patients ranging from ~0.7–2.7 mg/ml [21, 22, 36, 103, 106]. In injury, HA concentrations are lower and molecular weight distribution is shifted to lower molecular weight forms [6, 8, 14]. Transection of the rabbit anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) showed an association between decreased SF PRG4 concentrations and increased elastase activity, which can degrade PRG4 [27, 51]. Cellular PRG4 immunostaining and mRNA levels are decreased in degenerative cartilage compared to normal cartilage in an ovine meniscectomy model of OA [114], while in a bovine model, PRG4 mRNA expression and protein synthesis and secretion increased in explants from superficial regions of cartilage after 2 days following injurious compression [49]. Loss of HA from the joint is altered in a rabbit ACLT model, with a decrease in residence times of HA 7 days after ACLT [70].

The effects of injury on the concentration of PRG4 in SF appear variable (**Table 1.1**). Lubricin concentrations in the SF of patients with ACL injury were reported to be lower compared to SF from the uninjured contralateral knee during the first several months post-injury, and increasing to contralateral values by ~12 months [26]. However, SF lubricin concentrations in longitudinal samples from patients with recent severe knee injury decreased from the time of presentation (~15 days post-injury) to the time of arthroscopic surgery (~48 days post-injury) [19]. SF lubricin concentrations may be altered post-injury due to changes in the rates of synthesis, secretion, degradation, and/or loss from the joint, as well as changes in SF volumes. The effects of injury on lubricin synthesis and secretion have been studied with experimental models of cartilage mechanical injury. In a bovine model, lubricin

mRNA expression and protein synthesis and secretion increased in explants from superficial regions of cartilage with an intact articular surface after 2 days following injurious compression [49]. Further studies are needed to clarify the effects of injury on human SF lubricin concentrations. The absence of PRG4, both in patients with the genetic disease camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP) [68], an autosomal recessive disease with loss of function mutations in the PRG4 gene, and in *PRG4*$^{-/-}$ mice [88], results in cartilage degeneration and synovial hyperplasia.

Table 1.1: PRG4 concentrations in normal, osteoarthritic, and injured joints.

PRG4 Concentrations in Different Joint States [μg / ml]			References
Normal	**OA**	**Injury**	
~240		<<240 at early timepoints, approaching normal levels within 12 months	[26]
35±28 (n=3)	151±23 (n=21)		[78]
		390 ±160 at enrollment, t < 4 weeks, 15.2±7.2 days; 216±59 at surgery, 48±12 d after injury	[19]
287±32 (n=13)	95–426 (n=16)		[66]
159±36 (n=7)			[92]

1.3.3 Cytokines and Growth Factors

Cytokines and growth factors present in SF are important regulatory factors for the cell populations lining and within the synovial joint space, including chondrocytes and synovial cells [10, 42]. Regulatory molecules in SF may be derived from plasma or as secreted products of chondrocytes, synovial cells, and other cells within the SF or surrounding tissues. Cytokines may be categorized as pro-inflammatory or anti-inflammatory according to their predominant tissue-specific effects. Pro-inflammatory cytokines in SF include IL-1α, IL-1β, TNF-α, leukemia inhibitory factor (LIF), IL-6, IL-8, IL-17, and IL-18 [25, 34, 35]. Anti-inflammatory cytokines in SF include IL-4, IL-10, and IL-13. Growth factors including TGF-β1 and insulin growth factor 1 (IGF-I) are also present [18, 29, 85, 112], as are binding proteins, such as IL-1RA. The biological activity of soluble biochemical factors in regulating cell lubricant metabolism is a function of their concentrations and that of binding proteins, as well as the underlying signal transduction networks. Soluble biochemical regulators in joint may be synthesized locally by cells in the synovial joint. *In vitro*, tissue explants and isolated cells of synovium and cartilage are capable of secreting a number of factors, including: G-CSF [32], GM-CSF [2, 32], IL-1RA [9, 86], IL-6 [9, 32, 76], IL-8 [9, 32, 55], IL-10 [52], MIF [59], NO [73], OPG [9], PGE2 [32], and TGF-β1 [56] by synovial explants or isolated synoviocytes; IL-1β [9], IL-4 [9], IL-7 [9], IL-10 [9], IL-13 [9], IL-1RA [86], OPG [9], and TGF-β1 [111] by cartilage explants or isolated chondrocytes.

With joint injury and disease, the cytokine profile in SF is altered. Most cytokines and growth factors are at relatively low concentrations in normal SF, and are markedly elevated in joint injury and disease. Cytokines play an important role in disease pathogenesis and acceleration of joint destruction, and have attracted attention

as potential therapeutic targets, with some cytokine-directed therapies already in clinical use and others in clinical trials [67, 74]. Some pre-clinical studies have demonstrated efficacy in improving synovial joint lubrication. Blocking the effects of TNF-α with etanercept, for example, resulted in increased amounts of lubricin bound to cartilage and decreased sGAG release from cartilage compared with untreated groups in a rat ACL injury model of post-traumatic arthritis [28]. Binding proteins such as IL-1 receptor antagonist (IL-1RA) can play chondroprotective roles in the synovial joint and are found at relatively high levels in normal SF, with time-dependent changes in their concentrations after ACL injury [17, 69].

1.4 Articular Cartilage

Articular cartilage covering the ends of long bones provides friction-lowering, wear-resisting, and load-bearing functions in synovial joints under normal conditions. The organization of articular cartilage into three zones (superficial, middle, and deep), each with distinct composition and properties, facilitates the overall function of the tissue. The superficial zone comprises the 10–20% of the cartilage adjacent to the articular surface and contains chondrocytes that are one of the primary biosynthetic sources of PRG4 in the synovial joint [97]. In OA, early pathologic changes are apparent in the surface and superficial layers of articular cartilage [15].

The incidence of OA increases with age [16, 84], but the mechanisms underlying this relationship remain unclear. Aging is associated with chondrocyte and extracellular matrix changes and has been reviewed elsewhere [61-63]. Changes in chondrocytes include altered response to TGF-β due to an increase in the ratio of ALK1 to ALK5 expression, reduced response to IGF-I due to an increase in reactive oxygen species, and reduced Sirt1, all of which may promote a senescent secretory phenotype characterized by increased production of inflammatory cytokines, matrix metalloproteinases, and growth factors. Cell depletion, which may be region-dependent [110], impaired responses to extracellular stimuli, and deficiencies in homeostatic mechanisms such as autophagy [64] are additional age-associated changes in chondrocytes.

1.5 Proteoglycan 4 Lubricant Molecules

The *PRG4* gene encodes for superficial zone protein (SZP) and lubricin, which are mucinous glycoproteins with multiple O-linked β(1-3)Gal-GalNAc oligosaccharides that mediate boundary lubrication of articulating cartilage surfaces, acting to reduce friction and wear [45, 46, 94, 105, 107, 109]. SZP is a ~345 kDa glycoprotein synthesized and secreted by chondrocytes in the superficial zone of cartilage, and not from other depths of cartilage [96]. Lubricin is a ~220 kDa glycoprotein expressed by synovial fibroblasts and also present in SF [44, 47, 107]. Its physiological importance is suggested in patients and animals with mutations in the *PRG4* gene [43] that experience early onset joint abnormalities and failure [20, 48, 68, 88]. Furthermore, low concentrations of PRG4 have been associated with increased wear of the cartilage surface after knee injury [26, 27].

1.5.1 Structure

cDNA encodes a protein of 1,404 amino acids (human A isoform) with a somatomedin B homology domain, heparin-binding domains, multiple mucin-like repeats, a hemopexin domain, and an aggregation domain (**Fig. 1.3**). There are 3 consensus sequences for N-glycosylation and 1 chondroitin sulfate substitution site.

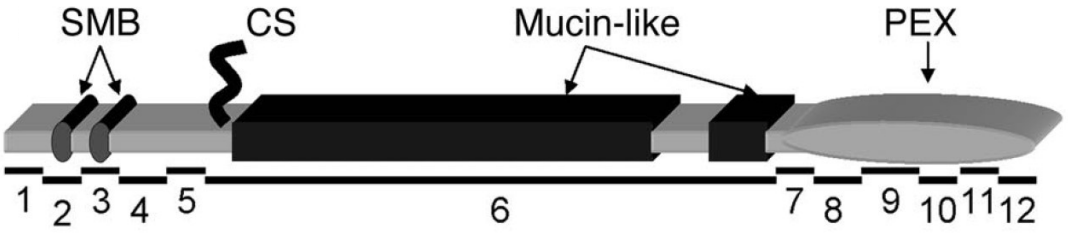


Figure 1.2: PRG4 structure. The *PRG4* gene contains 12 exons (labeled 1 – 12), with the amino-terminus at the left and the carboxyl-terminus at the right. *PRG4* has somatomedin B homology domains (SMB), multiple mucin-like repeats, a hemopexin (PEX) domain, and covalently linked chondroitin sulfate side chain (CS). (Figure is from [88]).

1.5.2 Function

PRG4 molecules play important roles in boundary lubrication, synovial and cartilage homeostasis, and immunomodulation. PRG4 reduces friction as assessed by friction tests using latex-glass [45], cartilage-glass [105], and cartilage-cartilage [94] interfaces. It prevents protein deposition on cartilage from SF by controlling adhesion-dependent synovial growth and inhibiting the adhesion of synovial cells to the cartilage surface. This is evident in *PRG4*-/- mice [88], which experience synoviocyte hyperplasia due to increased cell proliferation. Adhesion-dependent synoviocyte growth *in vitro* is inhibited by PRG4 [88]. PRG4 can also function as an immunomodulatory factor, as a PTH-responsive factor that regulates immune cells and some PTH actions on marrow hematopoietic progenitor cells. In studies of $PRG4^{-/-}$ vs. $PRG4^{+/+}$ mice, basal levels of SDF-1 protein expression, PTH-induced marrow SDF-1 protein expression, *IL-6* mRNA levels, PTH-induced expansion of marrow hematopoietic progenitor cells are all lower in mutant mice [79].

1.5.3 Regulation

Lubricant secretion by synovial joint cells, primarily synoviocytes and chondrocytes, can be influenced by chemical and mechanical stimuli. *In vitro*, biochemical stimuli, including TGF-β1[31, 50, 54, 93], a potent stimulus, and IL-1α [31, 50, 54, 93] and TNF-α [50, 54], potent inhibitors, are major regulators of cartilage PRG4 secretion, but other signaling molecules can also stimulate (BMP-7 [54], FGF-2 [54], IGF-I [31, 54, 93], PDGF [54], and oncostatin M [50]) or inhibit (retinoic acid [50]) PRG4 production (**Table 1.2**). Mechanical stimuli, including compression, shear, and continuous passive motion [80-82], also modulate PRG4 expression and secretion by fibroblast-like synoviocytes and chondrocytes. HA secretion, primarily by

fibroblast-like synoviocytes, is regulated by TGF-β1, IL-1β, and TNF-α [12]. In rat synovial membranes *in vitro,* HA secretion is regulated by TGF-β1, IL-1β, TNF-α, IFN-γ, IL-4, with responses to some cytokines different in synoviocytes compared to synovial explant cultures [42]. Synovium contains several cell types, including fibroblast-like synoviocytes and macrophage-like synoviocytes, and in diseased states, infiltrating immune cells, which may contribute to differences in responses at the cell and tissue explant level.

Table 1.2: Chemical regulators of cartilage PRG4 synthesis/secretion *in vitro*.

Chemical Factor	Effect on Cartilage PRG4 mRNA/Protein	References
TGF-β1	↑↑	[31, 50, 54, 93, 95]
IL-1α	↓↓	[31, 50, 54, 93, 95]
TNF-α	↓	[50, 54]
BMP7	↑	[54]
FGF-2	↑	[54]
IGF-I	↑	[31, 54, 93]
PDGF	↑	[54]
RA	↓	[50]
OSM	↑	[50]

1.6 Interactions between Joint Tissues and Fluid

The production and function of SF involves interactions between different joint tissues, cytokines, and the physical transport processes that connect all joint components. As new mechanistic details of SF, joint tissues, and cytokine effects are discovered, a comprehensive model of SF in health, injury, and disease will be needed to account for how the interactions between components give rise to joint function and behavior.

Interactions and regulatory influences between various synovial joint tissues play an important role in joint health and disease. The involvement of synoviocytes in the breakdown of cartilage in inflammatory arthritis has long been recognized [30], with the finding that synovial tissue culture medium contains a catabolic protein capable of inducing chondrocytes to degrade cartilage ECM [24]. More recently, the effects of RA synoviocyte metabolic products on chondrocyte gene expression have been studied *in vitro* by culturing chondrocytes in alginate beads suspended in conditioned medium from RA synovial fibroblasts, normal synovial fibroblasts, and anti-rheumatic drug treated synovial fibroblasts [4, 5], Chondrocytes cultured in RA synovial fibroblast conditioned medium, compared to normal synovial fibroblast conditioned medium, differentially expressed 110 genes, upregulating genes associated with immunological and catabolic processes and downregulating genes associated with cell proliferation and differentiation.

Responses to cytokines are specific to tissues, cell types, and culture conditions, and may be influenced by interactions between multiple cytokines. TGF-β1 generally upregulates HA secretion by normal synoviocytes [39, 42]. In chondrocytes and synoviocytes, TGF-β1 and IL-1β increased HA secretion,

individually and synergistically [87]. In rheumatoid fibroblastic synovial lining cells, IL-1β stimulates and TGF-β1 inhibits HA synthesis [53]. Three isoforms of hyaluronan synthase (HAS), the enzyme responsible for HA synthesis, have been identified, and HAS1 mRNA predominates in synovial cells, whereas HAS2 mRNA predominates in chondrocytes [87]. TGF-β1 has a differential effect on HAS in human fibroblast-like synoviocytes, upregulating HAS1 and downregulating HAS3 in a dose dependent manner [104]. Cytokines in SF modulate PRG4 as well, as TGF-β1 upregulates and IL-1β downregulates PRG4 gene expression and protein secretion in superficial zone cartilage, synovium, meniscus, and the anterior and posterior cruciate ligaments [58]. The effects of cytokines on cartilage matrix and chondrocyte metabolism have also been studied, with IL-1α, IL-1β, and TNF-α inhibiting proteoglycan synthesis and increasing proteoglycan degradation, while IGF-1 and TGF-β1 had the opposite effects [38].

1.7 Synovial Joint in Injury and Disease

The pathophysiology of the most common rheumatic disorders, OA and RA, and joint injury, involves multiple changes at the molecular, cellular, tissue, and organ levels [71] (**Figure 1**). The normal, healthy, synovial joint involves cartilage, synovium, SF, and other tissues functioning together (**Figure 1A**). OA is a complex, heterogeneous disease associated with alterations in cartilage degradation and repair, synovial inflammation, and increased production and SF levels of catabolic and pro-inflammatory cytokines (**Figure 1B**) [99]. The altered cytokine environment can enhance cartilage breakdown, which in turn may amplify synovial inflammation, leading to a cycle of events resulting in progressive joint destruction. OA develops progressively, but inflammatory flares can occur [99]. RA is a systemic inflammatory disease with features of synovitis, pannus formation, and SF volume increase, along with changes in the mass and concentration of plasma proteins, cytokines, and key proteases such as MMPs and their proenzyme activators and inhibitors (**Figure 1C**). Acute, traumatic joint injury, such as to the anterior cruciate ligament, result in a number of mechanical and biological changes over time that can contribute to the development of post-traumatic OA (**Figure 1D**) [3, 33, 65]. Post-traumatic OA is a subset of OA that is associated with an acute traumatic event. Some of the changes that may be involved in post-traumatic OA pathogenesis include increased cell death, increased levels of inflammatory cytokines and ECM-degrading enzymes, and decreased levels of lubricant molecules. Joint injury initiates events that, in many cases, lead to joint destruction, pain, and disability, with pathology and clinical symptoms that are similar to primary OA. In injury and disease states, analysis of

whole joint changes can provide an understanding of how joint pathophysiology arises from the interplay between component parts and contributes to the disease phenotype.

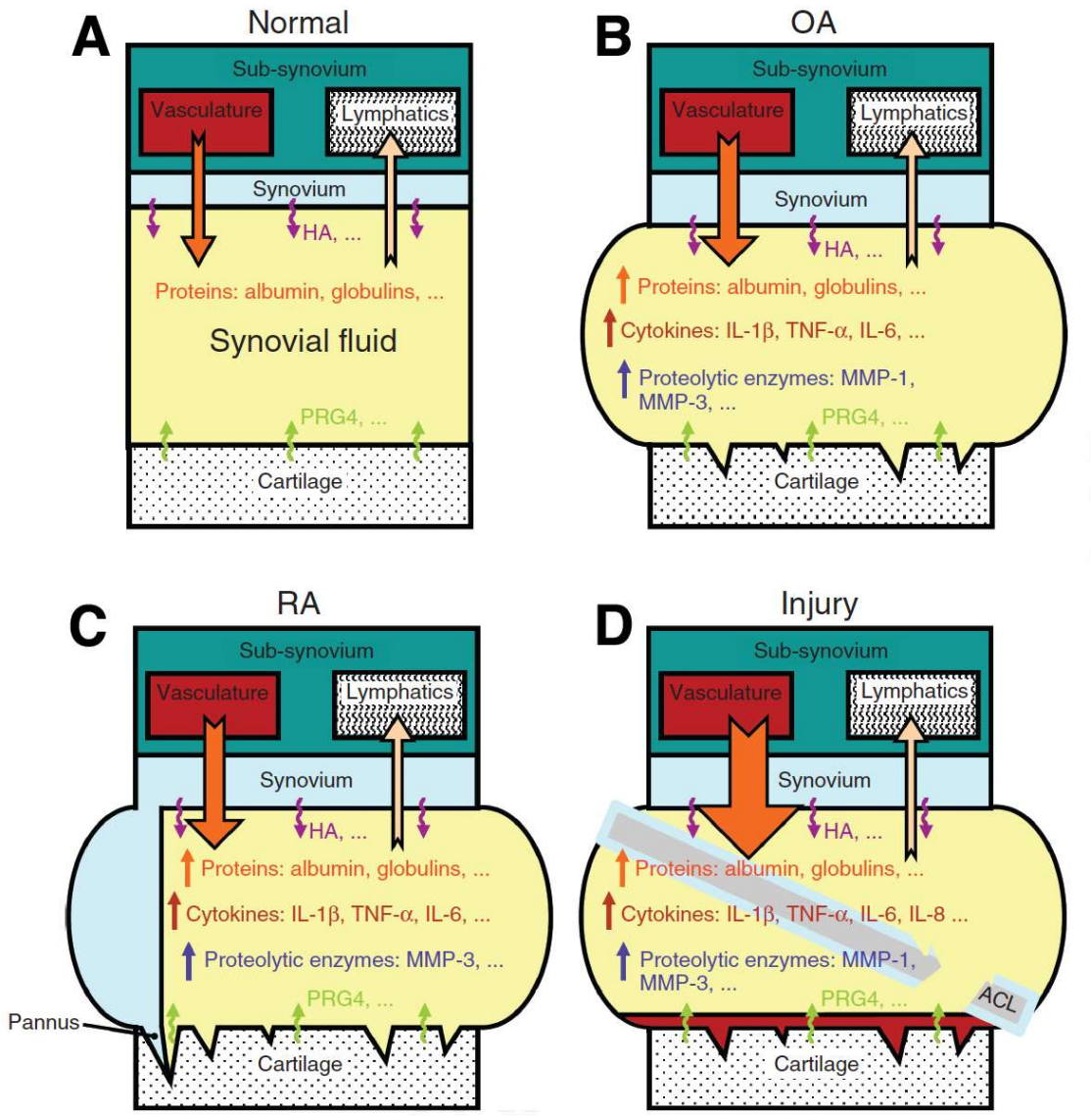


Figure 1.3: Synovial joint in health and osteoarthritis. Schematic view of synovial joint tissue changes in (**B**) OA, (**C**) RA, and (**D**) injury, compared to (**A**) a normal, healthy joint. Notched, outlined arrows represent fluid flows from vasculature to SF, and from SF to lymphatics. Undulating arrows represent secretion of lubricant molecules HA and PRG4 into SF. The relative sizes of arrows indicate relative magnitudes of flows and secretions. For example, increased flow from the vasculature to SF in (**D**) injury compared to (**A**) normal is represented by an orange, notched, outlined arrow that is larger in (**D**) injury compared to (**A**) normal. Up-arrows indicate increases in concentrations of the various substances. (Figure adapted from [41]).

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CHAPTER 2:

AGE-DEPENDENT TRANSFORMING GROWTH FACTOR-β1 REGULATION OF PROTEOGLYCAN 4 SECRETION BY HUMAN KNEE ARTICULAR CARTILAGE

2.1 Abstract

Objective: Aging and PRG4 lubricant deficiency are risk factors for osteoarthritis, but their relation is unknown. The objectives of this study were to assess in normal human cartilage the age-associated changes in constitutive and TGF-β1-stimulated (1) *PRG4* gene expression, (2) PRG4 protein secretion, and (3) PRG4+ chondrocyte density.

Methods: Explants from macroscopically normal human cartilage from lateral femoral condyles of donors (n=45, range=20–88 yr) were processed for constitutive analyses or incubated in medium±TGF-β1. The dose-response and time-course of TGF-β1-stimulated PRG4 secretion were determined. The age-dependent TGF-β1 regulation of PRG4 expression, secretion, and localization was assessed. *PRG4* gene expression and PRG4 protein localization were assessed by qPCR and IHC,

respectively. PRG4 protein secretion into medium was assessed by ELISA and Western blot.

Results: Constitutive cartilage *PRG4* mRNA and PRG4 protein secretion were lower with increasing age. TGF-β1 stimulation of cartilage *PRG4* gene expression and PRG4 protein secretion were blunted with age (–81% and –17% per decade, respectively). The density of PRG4+ chondrocytes in cartilage was lower with age, constitutively and after TGF-β1 stimulation (–15% per decade), whereas the total number of chondrocytes in the superficial zone was not altered with age.

Conclusion: Aging results in diminished PRG4 secretion by normal human femoral condyle cartilage, predominantly due to a marked reduction in the number of PRG4+ chondrocytes, but not overall cellularity. The age-associated decrease in cartilage constitutive and TGF-β1-stimulated PRG4 production may be related to the age-associated predisposition to cartilage degeneration and osteoarthritis.

2.2 Introduction

Articular cartilage provides friction-lowering, wear-resisting, and load-bearing functions in synovial joints. The organization of articular cartilage into three zones (superficial, middle, and deep), each with distinct composition and properties, facilitates the overall function of the tissue. The superficial zone, comprising the 10–20% of the cartilage adjacent to the articular surface, consists of chondrocytes that are flattened and elongated in the direction parallel to the surface and embedded in an extracellular matrix composed primarily of densely organized collagen fibrils [32]. In osteoarthritis (OA), early pathologic changes are apparent in the surface and superficial layers of articular cartilage [6].

Within the cartilage superficial zone resides a population of chondrocytes that serves as a major biosynthetic source of proteoglycan 4 (PRG4) in the synovial joint [47]. The term PRG4 is used here to refer to the proteoglycan products that are encoded by the *PRG4* gene and that have been named superficial zone protein (SZP) and lubricin. SZP is mucinous glycoprotein product, with an apparent MW of ~345 kDa, of the *PRG4* gene that is synthesized and secreted primarily by chondrocytes in the superficial zone. SZP localizes at the articular surface and is secreted into the culture medium by cartilage slices containing the superficial zone [46]. The *PRG4* gene [17] also encodes lubricin, a ~220 kDa glycoprotein, expressed by synovial fibroblasts and isolated from synovial fluid [18, 21, 52]. The importance of PRG4 is manifest in patients with camptodactyly-arthropathy-coxa vara-pericarditis syndrome, who have an autosomal recessive mutation in the *PRG4* gene and experience early

onset joint failure [30]. PRG4 provides several functions to help maintain homeostasis in the synovial joint, including lubrication of the cartilage surfaces and prevention of synovial overgrowth [41]. While PRG4 normally functions to lubricate articulating cartilage surfaces [20, 44, 51], low concentrations of PRG4 are associated with increased wear of cartilage after knee injury [10, 11]. Also, some patients with knee OA have synovial fluid with low PRG4 concentration and boundary-lubricating function, which is restored *in vitro* upon PRG4 supplementation [28].

TGF-β1 is a growth factor with pleiotropic effects in the synovial joint, acting to stimulate chondrocyte proliferation and matrix synthesis, synovial proliferation, fibrosis, and expression of some OA-related genes, and osteophyte formation [4, 26, 40]. Together with other biochemical stimuli, such as IL-1α, IL-1β, IGF-I, and TNF-α [5, 13, 43], and mechanical stimuli, such as compression [35], shear [34], and continuous passive motion [36], TGF-β1 modulates chondrocyte *PRG4* expression and PRG4 secretion. TGF-β1 is a potent stimulus for PRG4 secretion by chondrocytes, both in cell [13, 23, 25] and cartilage explant [22, 43] culture. The concentration of TGF-β1 in synovial fluid is elevated in diseases including OA [12] and may be increased by intra-articular injection of platelet-rich plasma [42].

While the incidence of OA increases with age [7, 37], the relationship between aging, cartilage degeneration, and OA remains to be fully elucidated. A number of age-related changes occur in cartilage and chondrocytes, and in the superficial zone of cartilage. These changes include site-specific decreases in cellularity and alterations in responsiveness to growth factors and expression of transcriptional regulators [14, 16, 29, 49, 53, 55]. Despite the strong association between aging and OA, and the link

between altered PRG4 levels and cartilage degeneration, the relationship between aging, *PRG4* expression, and PRG4 secretion in human cartilage is unknown. With aging, decreased PRG4 secretion may be an age-associated risk factor for the development of OA. Within the knee joint, superficial zone cartilage cellularity is largely maintained with aging in the lateral femoral condyle (LFC), but not in the medial femoral condyle [55]. Thus, LFC cartilage offers the opportunity to examine whether normal aging leads to altered chondrocyte responsiveness and PRG4 secretion.

The hypothesis of this study was that with normal human cartilage aging, there is a decline in PRG4 secretion, associated with diminished chondrocyte response to TGF-β1. The aims were to assess, in normal human knee articular cartilage, the effect of donor age on (1) constitutive and (2) TGF-β1-stimulated *PRG4* gene expression, PRG4 secretion, and density of total and PRG4-immunolocalized (PRG4+) cells.

2.3 Materials and Methods

2.3.1 Sample Selection

Cartilage samples were selected from cadaveric donors that exhibited normal aging (20–88 yr, n=45) and distributed across Young (20–40 yr), Middle (41–60 yr), and Old (≥61 yr) age groups. Human knee joints were obtained from tissue banks with approval of Human Subjects Committees and processed within 72 hours post-mortem. Cartilage surfaces of the femoral condyles, trochlea, and tibial plateau were inspected macroscopically and graded using the International Cartilage Repair Society classification system [1]. Joints exhibiting OA, based on the presence of either osteophytes or subchondral bone exposure, were excluded. In addition, only cartilage without macroscopic evidence of erosion (ICRS Grades 0–1) was used.

2.3.2 Cartilage Explant Harvest

Cartilage disks, 3 mm diameter and containing the intact articular surface, were explanted (3–10 per donor LFC) using sterile technique. For *PRG4* gene expression and PRG4 protein secretion analyses, cartilage disks, ~0.4 mm thick, were used. For depth-associated immunohistochemical (IHC) analyses, cartilage disks, ~1.5 mm thick, from the LFC central region were used. For cell content estimates in the superficial zone, cartilage disks, ~0.2 mm thick, were used. All explants from an individual donor were distributed randomly amongst experimental groups and either processed directly for constitutive analyses or incubated.

2.3.3 Tissue Culture

Cartilage was incubated for 6 days in basal medium±rhTGF-β1 (PeproTech) at doses specified below, at 37 ºC and 5% CO_2. Basal medium consisted of Dulbecco's Modified Eagle Medium, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid,

0.1 mM MEM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 25 μg/ml ascorbic acid, and 0.01% bovine serum albumin. Medium conditioned by incubated cartilage was collected and replaced with fresh medium of the same type every 2 days. Explants for IHC analyses were incubated for 6 days, then overnight (12-16 hr) in fresh medium supplemented with 0.1 μM monensin to inhibit PRG4 secretion, enhance intracellular accumulation, and separate cells into two types, immunostaining negative or positive for PRG4 (Schumacher+, JOR 1999, Klein+, OAC 2003, Schmidt+, OAC 2008).

2.3.4 Experimental Design

Aim 1: Constitutive *PRG4* Gene Expression and Quantification of Total and PRG4-expressing Cells. To determine if constitutive *PRG4* gene expression and the number and depth-variation of PRG4+ are age-dependent, cartilage from donors spanning a wide age range were analyzed fresh. For constitutive *PRG4* gene expression, explants from n=11 donors (22–88 yr, 4*M*, 7*F*) were examined by qPCR. For the number of chondrocytes expressing PRG4, overall and as a function of depth from the articular surface, explants from n=13 donors (28–84 yr, 8*M*, 5*F*) were examined by IHC. To determine if the overall density of chondrocytes in the superficial zone varied with age, DNA content in explants from n=17 donors (23–79 yr, 10*M*, 7*F*), different from those studied for PRG4 gene expression, was determined biochemically and normalized to wet weight.

Aim 2: TGF-β1-Stimulated *PRG4* Gene Expression, PRG4 Protein Secretion, and Quantification of PRG4-expressing Cells. To determine the age-dependence of TGF-β1 stimulation of *PRG4* gene expression, PRG4 protein secretion, and the number of PRG4-expressing cells, cartilage from donors spanning a wide age

range were incubated in medium±TGF-β1 for 6 days. The incubated cartilage was analyzed for *PRG4* gene expression (22–88 yr, n=12, 4*M*, 8*F*), and conditioned medium (days 4–6) was analyzed for PRG4 protein. ***TGF-β1 Dose-response***. Explants (n=8, 3*M*, 5*F*) from Young (n=4) and Old (n=4) donors were incubated in medium alone, or supplemented with 0.3, 1, 3, or 10 ng/ml TGF-β1, and medium conditioned by cartilage (days 2–6) was analyzed for PRG4 protein. The TGF-β1 dose (10 ng/ml) that maximally stimulated PRG4 secretion was used for later experiments. ***TGF-β1 Time-course***. Explants (n=14, 5*M*, 9*F*) from Young (n=7) and Old (n=7) donors were incubated in medium±10 ng/ml TGF-β1 for 6 days, with medium±TGF-β1 replenished every 2 days and analyzed for PRG4 secretion. ***Proportion of PRG4 in Conditioned Medium that is Inhibited by Cycloheximide***. To determine the proportion of PRG4 released into medium that is dependent on active translation of *PRG4*, explants from young donors (22–39 yr, n=6, 3*M*, 3*F*) were incubated in the presence or absence of TGF-β1 and/or cycloheximide (CHX). ***Age-dependence of TGF-β1-stimulated Cartilage PRG4 Secretion***. Explants from donors (20–88 yr, n=26, 11*M*, 15*F*) were incubated in medium±10 ng/ml TGF-β1 for 6 days. *PRG4* gene expression in cartilage (n=12), and PRG4 protein in conditioned medium from days 4–6 (n=26), were assessed. To distinguish between the possibilities that age-dependent PRG4 secretion was the result of age-dependent changes in cellular response versus altered cellularity, explants were analyzed by IHC. The number of PRG4+ chondrocytes, total and as a function of depth from the articular surface, was quantified.

2.3.5 *PRG4* Gene Expression

Cartilage was stored in RNAlater (Qiagen) to preserve RNA, then snap-frozen in liquid nitrogen and pulverized. RNA extraction was performed using RNeasy (Qiagen). First strand cDNA was synthesized using SuperScript First-Strand Synthesis

System (Invitrogen). qPCR amplification was performed using commercial primers targeting exon 3 (SABiosciences) and rRNA 18S (r18s) to quantify the level of *PRG4* expression [9] normalized to r18s expression.

2.3.6 PRG4 Protein Secretion

PRG4 secreted into conditioned medium was quantified by indirect ELISA [24, 45] and characterized by Western blot [2], essentially as previously described, except using the polyclonal E-19 primary antibody that recognizes an epitope within the 1100–1150 amino acid residues in PRG4 (Santa Cruz Biotechnology) for both ELISA and Western blot, as noted below. ***PRG4 ELISA***. Conditioned medium was diluted serially, adsorbed to ELISA plates, reacted with polyclonal goat antibody E-19 to human PRG4, horseradish peroxidase-conjugated anti-goat IgG rabbit secondary antibody, and ABTS substrate, with PBS-0.1% Tween washes between each step. PRG4 secretion was expressed normalized to cartilage surface area and number of days of incubation (μg/[cm^2·day]). ***PRG4 Western blot***. To characterize the size distribution of immunoreactive PRG4, conditioned medium was fractionated on 2% agarose gel, transferred to polyvinylidene difluoride membrane, and reacted with the polyclonal goat antibody E-19 to PRG4, at 0.5 μg/ml, and a donkey anti-goat secondary antibody, at 1:100,000 dilution. Bands were visualized by ECL-Plus detection and digital scanning with a STORM 840 Imaging System (Molecular Dynamics). Only narrow vertical regions, encompassing the standard, of the Western blot are shown because there was little to no immunoreactivity elsewhere (data not shown). Samples containing known amounts of human PRG4, standardized against human PRG4 purified by anion-exchange chromatography and YM100 concentration, analyzed for purity by electrophoresis, and quantified by bicinchoninic acid protein assay, served as standards for ELISA and Western blot. For the ELISA, these samples

were assayed in twofold dilutions ranging from 4–500 ng/ml to generate a sigmoidal standard curve.

2.3.7 Inhibition of PRG4 Secretion by Cycloheximide

Explants were incubated in medium 1) alone, 2) with 10 ng/ml TGF-β1, 3) with 0.1 mM CHX to inhibit protein synthesis or 4) with TGF-β1 and CHX. CHX treatment was initiated at day 4, to examine the extent that PRG4 released into the medium at days 4–6 of culture could be attributed to active translation of *PRG4*.

2.3.8 PRG4 Protein Immunolocalization [24]

Briefly, 5 μm thick cryosections normal to the articular surface were prepared, reacted with the polyclonal goat antibody E-19, and localized by peroxidase-based detection, followed by staining with propidium iodide (0.02 mg/ml in PBS) for visualization of cell nuclei. Sections probed with non-specific goat IgG antibody served as negative controls. Stained samples were viewed by transmitted light and then fluorescence microscopy, digitized, and analyzed manually to identify PRG4+ cells and automatically with a particle-detecting image-processing program, essentially as described previously [39], to identify PI+ cell nuclei with the experimental groups blinded. The majority of PRG4+ cells co-localized with propidium iodide fluorescence (84%±13%, n=8 representative samples, e.g. Fig. 1C-E), consistent with diffuse PRG4+ staining in the cytoplasm and localized PI+ staining of the nucleus. The depth-associated variation in chondrocytes that were PRG4+ was determined in a representative 0.60 mm wide x 1.35 mm (area=0.81 mm^2) deep region of each section. PRG4+ chondrocytes were identified manually with the experimental groups blinded. The number of PRG4+ cells in successive 100 μm layers below the articular surface and the total number of PRG4+ cells were quantified.

2.3.9 Cartilage Cellularity

Explants were weighed wet, solubilized with proteinase K, and portions reacted with PicoGreen® to determine DNA content [31]. DNA was converted to cell number using a conversion factor of 7.3 pg DNA / human chondrocyte [49]. DNA content and cell numbers were normalized to wet weight.

2.3.10 Statistical Analysis

Data are presented as mean±SEM, or geometric mean (95% confidence interval (CI)) for log-transformed data, unless otherwise specified. Data that ranged over an order of magnitude were $\log_{10}$ transformed to improve normality and homoscedasticity. The effects of TGF-β1 dose (0, 0.3, 1, 3, or 10 ng/ml) and age group (Young or Old) on PRG4 secretion were assessed by two-way repeated measures ANOVA with TGF-β1 dose as within-subject factor. The effects of duration of culture (0–2, 2–4, or 4–6 days), age group (Young or Old), and TGF-β1 (0 or 10 ng/ml) on PRG4 secretion was assessed by three-way repeated-measures ANOVA, with TGF-β1 and days of culture as within-subject factors. Dunnett's post-hoc test was used to compare PRG4 secretion at each TGF-β1 dose to that without TGF-β1, and PRG4 secretion at days 2–4 and 4–6 to that at days 0–2. The effect of age group (Young, Middle, or Old) on constitutive *PRG4* gene expression was assessed by one-way ANOVA followed by Tukey's post-hoc test. The age-dependence of *PRG4* gene expression, PRG4 secretion, and area-density of PRG4+ cells, in explants cultured with and without TGF-β1 were analyzed by univariate linear regression. For measures fitted to regression models (PRG4 secretion and area-density of PRG4+ cells), percent change per decade was determined from slopes of regression lines and the regression equation evaluated for x=21 yr. For log-transformed measures fitted to regression models (*PRG4* gene expression), percent change per decade was determined from back-transforms of slopes of regression lines. Counts of PRG4+ cells were square-root

transformed prior to two-way repeated-measures ANOVA to test the effects of age group (between-subject factor) and depth from articular surface (within-subject factor). At each depth, ANOVA was used to assess the effect of age group on the quantity of PRG4+ cells. When age group had a significant effect, Tukey's post-hoc test was used to compare the counts of PRG4+ cells between different age groups. Matching was effective for all repeated-measures ANOVAs as confirmed by lower significance values compared to the same data analyzed by ordinary ANOVAs. Statistical significance level was set at $\alpha=0.05$. Analyses were performed with SYSTAT 10.2 (Systat Software).

2.4 Results

2.4.1 Constitutive *PRG4* Gene Expression and Number of PRG4+ Cells are Lower with Aging

Constitutive *PRG4* expression was at levels that were markedly higher in cartilage from Young donors than Middle ($p<0.001$) and Old ($p<0.001$) donors (**Fig. 2.1A**). The number of chondrocytes immunostaining for PRG4 (PRG4+) was lower (slope=–1.35 [–2.20, –0.50] cell/0.81 mm^2/yr, –12.4% [–20.2%, –4.6%] per decade relative to a 21 yr, $R^2=0.53$, $p<0.01$) with increasing donor age (**Fig. 2.1B**). In contrast, superficial zone cartilage cellularity was not altered substantially with donor age (slope=–0.10 [–0.36, 0.15] cell/yr, –4.1% [–14.2%, 5.9%] per decade, $R^2=0.05$, $p=0.40$). Representative low and high power images of PRG4 immunostaining for (**Fig. 2.1C**) 36, (**Fig. 2.1D**) 52, and (**Fig. 2.1E**) 79 yo donors, as well as for samples reacted with non-specific IgG primary antibody (**Fig. 2.1F**), indicated staining specificity and the relative number of PRG4+ cells as a function of depth from the articular surface. The differences in the area density of PRG4+ cells between age groups was most substantial near the surface, with 31 PRG4+ cells/0.06 mm^2 in Young compared to 5 PRG4+ cells/0.06 mm^2 in Old ($p<0.01$) donors (**Fig. 2.1G**).

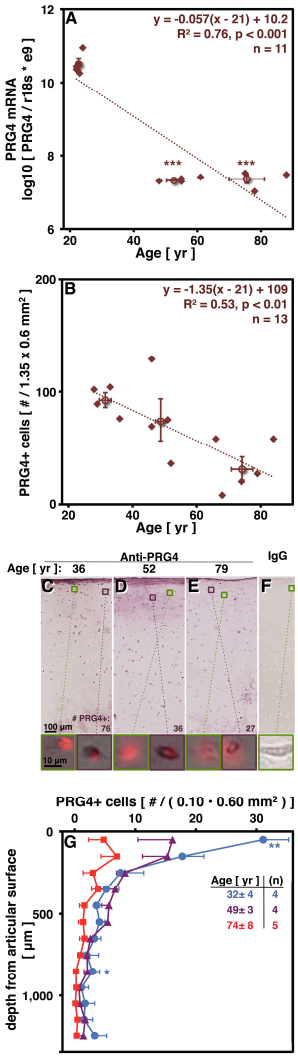


Figure 2.1: *PRG4* gene expression and PRG4 protein localization in fresh explants of human cartilage from lateral femoral condyles. (**A**) *PRG4* gene expression in fresh explants (n=11 donors). (**B**) Quantification of PRG4+ cells by immunolocalization in a 1.35 mm x 0.60 mm area of vertical sections of explants (n=13 donors). (**C-E**) PRG4 immunolocalization in explants from a (**C**) 36, (**D**) 52, and (**E**) 79 yo donors, with (**F**) IgG control from the 52 yo donor. The number of PRG4+ cells in each 0.60 mm x 1.35 mm (area=0.81 mm^2) section is shown in the lower right of each frame. Typical higher magnification images (merged transmitted light and propidium iodide fluorescence for samples probed for PRG4, transmitted light alone for IgG control) of cells scored as PRG4+ (purple outlines) and PRG4- (green outlines) are shown below. (**G**) Quantification of PRG4+ cells as a function of depth from the articular surface, grouped by age (blue=20–40, purple=41–60, and red=≥61 yr). (**A,B**) Mean (•) and SEM (+) denoted within each age group of 20–40, 41–60, ≥61 yr. *p<0.05, **p<0.01, ***p<0.001 compared to Young group.

2.4.2 PRG4 Protein Secretion is Stimulated by TGF-β1 in a Dose-dependent Manner

Cartilage PRG4 secretion was affected by TGF-β1 treatment ($p<0.05$) and age group ($p<0.001$) independently and also interactively ($p<0.01$). TGF-β1 treatment had a stimulatory effect in Young ($p<0.01$) and an effect approaching significance in Old ($p=0.055$) donors (**Fig. 2.2A**). In Young donors, all doses of TGF-β1 elicited a robust PRG4 secretion response over basal levels, with the strongest responses at 3 and 10 ng/ml TGF-β1 (+499% and +404%, each $p<0.001$) and an apparent plateau.

2.4.3 TGF-β1 Prevents the Decrease in PRG4 Protein Secretion During Cartilage Culture

Over 6 days, PRG4 secretion by cartilage incubated in basal medium decreased during the six days of culture, for both Young ($p<0.001$) and Old ($p<0.05$) donors, but not for explants incubated in medium with 10 ng/ml TGF-β1 ($p=0.55$ for Young, $p=0.17$ for Old) (**Fig. 2.2B**). Western blot of conditioned medium samples showed a distinct immunoreactive band at an apparent molecular weight of ~345 kDa, consistent with PRG4 standard, with non-specific goat IgG antibody serving as negative controls (**Fig. 2.2C**). Band intensities were qualitatively consistent with PRG4 secretion as determined by ELISA.

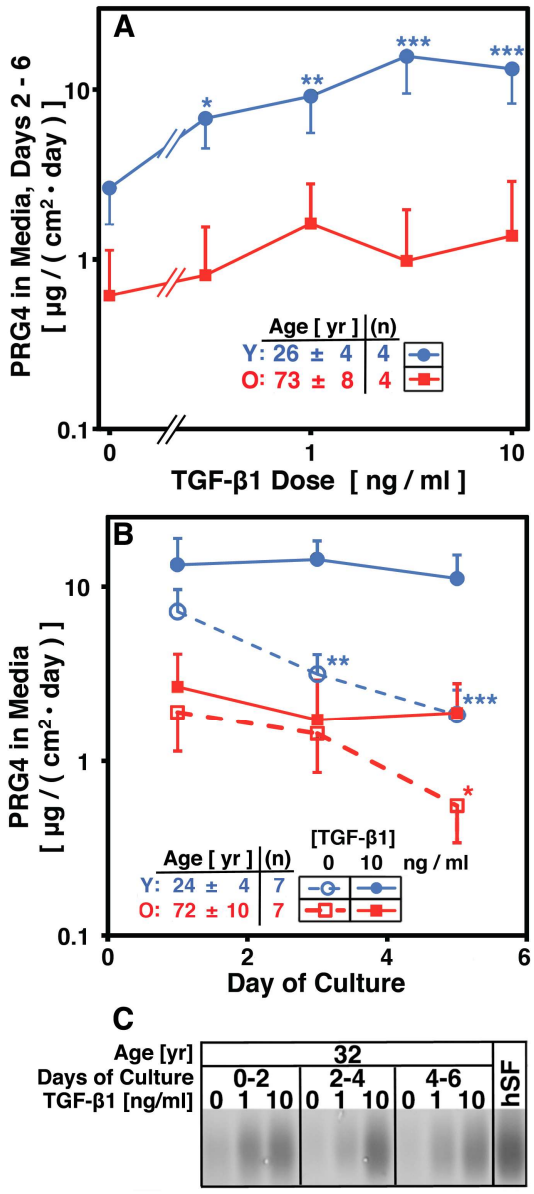


Figure 2.2: Dose-response and time-course of PRG4 secretion by explants incubated in medium±TGF-β1. (**A**) PRG4 secretion by cartilage (days 4–6 of culture) from young (●) and old (■) donors incubated in medium with increasing doses of TGF-β1 (n=4 young, n=4 old donors). (**B**) PRG4 secretion by explants from young (n=7, ○,●) and old (n=7, □,■) donors incubated in medium in the absence (○,□) or presence (●,■) of 10 ng/ml TGF-β1 over 6 days of culture. (**C**) Western blot of conditioned medium from explants of a 32 yo donor incubated in medium with increasing doses of TGF-β1 over 6 days, and of a human synovial fluid (hSF) sample serving as positive control. *p<0.05, **p<0.01, ***p<0.001 relative to without TGF-β1 or days 0–2.

2.4.4 TGF-β1-stimulated Cartilage PRG4 Release into Medium is Inhibited by Cycloheximide

PRG4 released into conditioned medium by cartilage explants during days 4–6 of incubation was modulated by TGF-β1 ($p<0.05$) and CHX ($p<0.01$) in an interactive manner ($p<0.05$). TGF-β1-stimulated PRG4 release by explants was substantially lower with CHX treatment compared to without (0.9±0.3 vs. 6.4±3.4 μg/[cm^2·day], $p<0.05$). PRG4 release by cartilage explants incubated in basal conditions was low, both in the presence or absence of CHX (0.7±0.2 and 0.7±0.3 μg/[cm^2·day]).

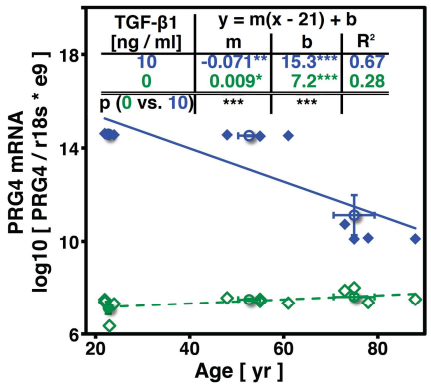
2.4.5 TGF-β1 Stimulation of *PRG4* Gene Expression and PRG4 Protein Secretion are Age-dependent

The extent of TGF-β1 stimulation of cartilage *PRG4* gene expression decreased with age (**Figs. 2.3A,B**). Log-transformed *PRG4* gene expression after 6 days of culture with TGF-β1 stimulation was lower with increasing age (slope=–0.071 [–0.107, –0.035] $\log_{10}$(PRG4/r18s*e9)/yr, –81% [–91%, –56%] per decade R^2=0.67, $p<0.01$), while that after 6 days of basal culture was low and steady with age (slope=0.009 [0.000, 0.018] $\log_{10}$(PRG4/r18s*e9)/yr, 23% [0.2%, 51.1%] per decade, R^2=0.28, $p<0.05$). TGF-β1 stimulation of *PRG4* gene expression decreased with age, as indicated by the difference in slopes ($p<0.001$) and intercepts ($p<0.001$) of the two regression lines. The induction of *PRG4* expression by TGF-β1 was over markedly higher in cartilage from Young ($p<0.01$) and Middle ($p<0.05$) donors than Old donors (**Fig. 2.3B**).

The extent of TGF-β1 stimulation of cartilage PRG4 secretion also decreased with age (**Figs. 2.3C,D**). PRG4 secretion during days 4–6 of culture was lower with increasing age for explants stimulated with TGF-β1 (slope=–0.23 [–0.35, –0.10] μg/(cm^2·day)/yr, –17% [–26.0%, –7.7%] per decade, R^2=0.38, $p<0.001$) and without

(slope=–0.03 [–0.06, –0.01] μg/(cm^2·day)/yr, –15% [–22.9%, –7.0%] per decade, R^2=0.26, $p<0.01$). The TGF-β1 stimulation of PRG4 secretion was reduced with age, as indicated by the difference in slopes ($p<0.01$) and intercepts ($p<0.001$) of the two regression lines. The increase in PRG4 secretion by TGF-β1 was substantially higher in Young (7.6 μg/(cm^2·day)) than Old (1.4 μg/(cm^2·day)) donors ($p<0.05$) (**Fig. 2.3D**).

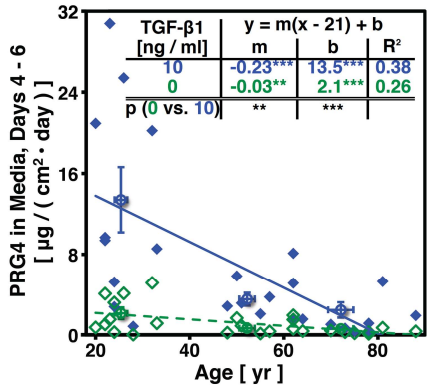
A



B

Age Group Range [yr]	Young 20–40	Middle 41–60	Old ≥61
Age [yr] n	23±1 4	53±4 3	75±10 5
Fold Induction of *PRG4*[a] (CI)	28 x 10^6 (16–50) x 10^6	10 x 10^6 (9–11) x 10^6	3.2 x 10^3 ** (0.4–26.8) x 10^3

C



D

Age Group Range [yr]	Young 20–40	Middle 41–60	Old ≥61
Age [yr] n	25±4 10	52±4 5	72±9 11
Increase in PRG4[b] (CI) [µg / (cm^2 · day)]	7.6 (3.8–14.5)	2.8 (2.1–3.6)	1.4 * (0.6–2.4)

Figure 2.3: Age-dependence of TGF-β1 effects on *PRG4* gene expression and PRG4 protein secretion. (**A**) *PRG4* gene expression and (**C**) PRG4 protein secretion by explants incubated in medium in the absence (green) or presence (blue) of 10 ng/ml TGF-β1, with mean (•) and SEM (+) denoted within each age group of 20–40, 41–60, ≥61 yr. **p<0.01, ***p<0.001. (**B**) Fold induction of *PRG4* gene expression and (**D**) increase in PRG4 protein secretion in age groups, with data presented as arithmetic mean±SD or geometric mean (95% CI). [a]Induction is the ratio of *PRG4* expression in explants stimulated with TGF-β1 to without. [b]Increase is the difference between PRG4 secretion by explants stimulated with TGF-β1 to without. *p<0.05, **p<0.01 relative to Young group.

2.4.6 Number of PRG4+ Cells after TGF-β1 Stimulation is Lower with Aging

With increasing age, the number of PRG4+ cells in cartilage incubated with TGF-β1 was lower (slope=–2.00 [–3.07, –0.94] cell/0.81 mm^2/yr, –15.0% [–22.9%, –7.0%] per decade, R^2=0.61, p<0.01) (**Figs. 2.4E-G, 2.5B**), and a similar trend was evident for cartilage incubated in basal medium (slope=–0.90 [–1.84, 0.04] cell/0.81 mm^2/yr, –11.9% [–24.3%, 0.5%] per decade, R^2=0.29, p=0.059) (**Figs. 2.4A-C, 2.5A**). Identification of PRG4+ cells appeared specific, based on the lack of immunostaining using non-specific IgG primary antibody (**Figs. 4D, H**). There was marked depth-associated variation of PRG4+ cells after culture, both for cartilage incubated in basal medium (p<0.001) (**Fig. 2.5C**) or in medium with TGF-β1 (p<0.001) (**Fig. 2.5D**). For cartilage incubated in medium with TGF-β1, the largest absolute differences between age groups in the area-density of PRG4+ cells in explants was in the layers nearest the articular surface (**Fig. 2.5D**). In cartilage treated with TGF-β1, at 0–100 μm below the articular surface, there were 29 PRG4+ cells/0.06 mm^2 in explants from Young compared to 5 PRG4+ cells/0.06 mm^2 in Old (p<0.01) donors, and at 100–200 μm, there were 19 vs. 3 PRG4+ cells/0.06 mm^2 in young vs. old donors (p<0.05). In cartilage incubated in basal medium (without TGF-β1), at 0–100 μm below the articular surface, the area-density of PRG4+ cells did not differ significantly between age groups (**Fig. 2.5C**).

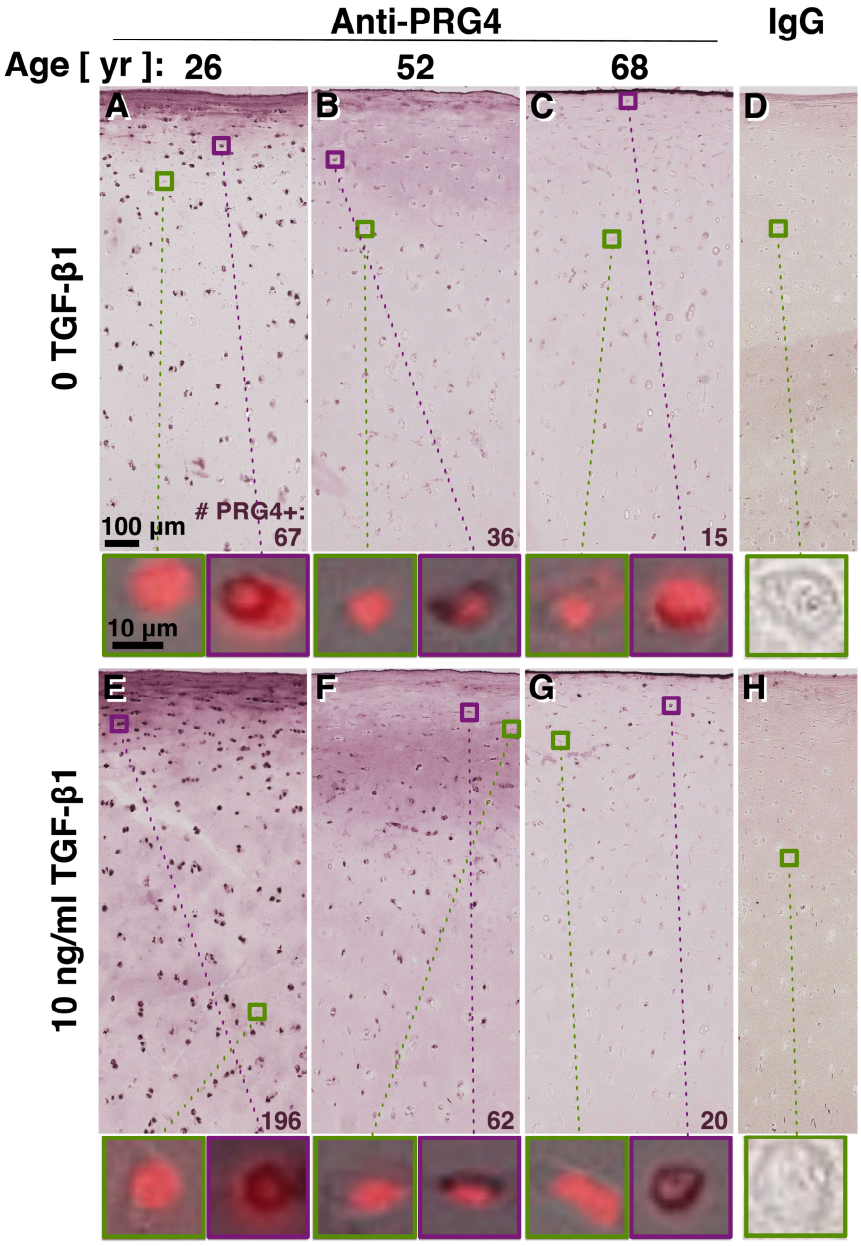


Figure 2.4: PRG4 protein immunolocalization in explants from a (A,E) 26, (B,F) 52, and (C,G) 68 yo donors incubated in medium in the (A-D) absence or (E-H) presence of 10 ng/ml TGF-β1 for 6 days. The number of PRG4+ cells in each 0.60 mm x 1.35 mm (area=0.81 mm^2) section is shown in the lower right of each frame. Typical higher magnification images (merged transmitted light and propidium iodide fluorescence for samples probed for PRG4, transmitted light alone for IgG control) of cells scored as PRG4+ (purple outlines) and PRG4- (green outlines) are shown below.

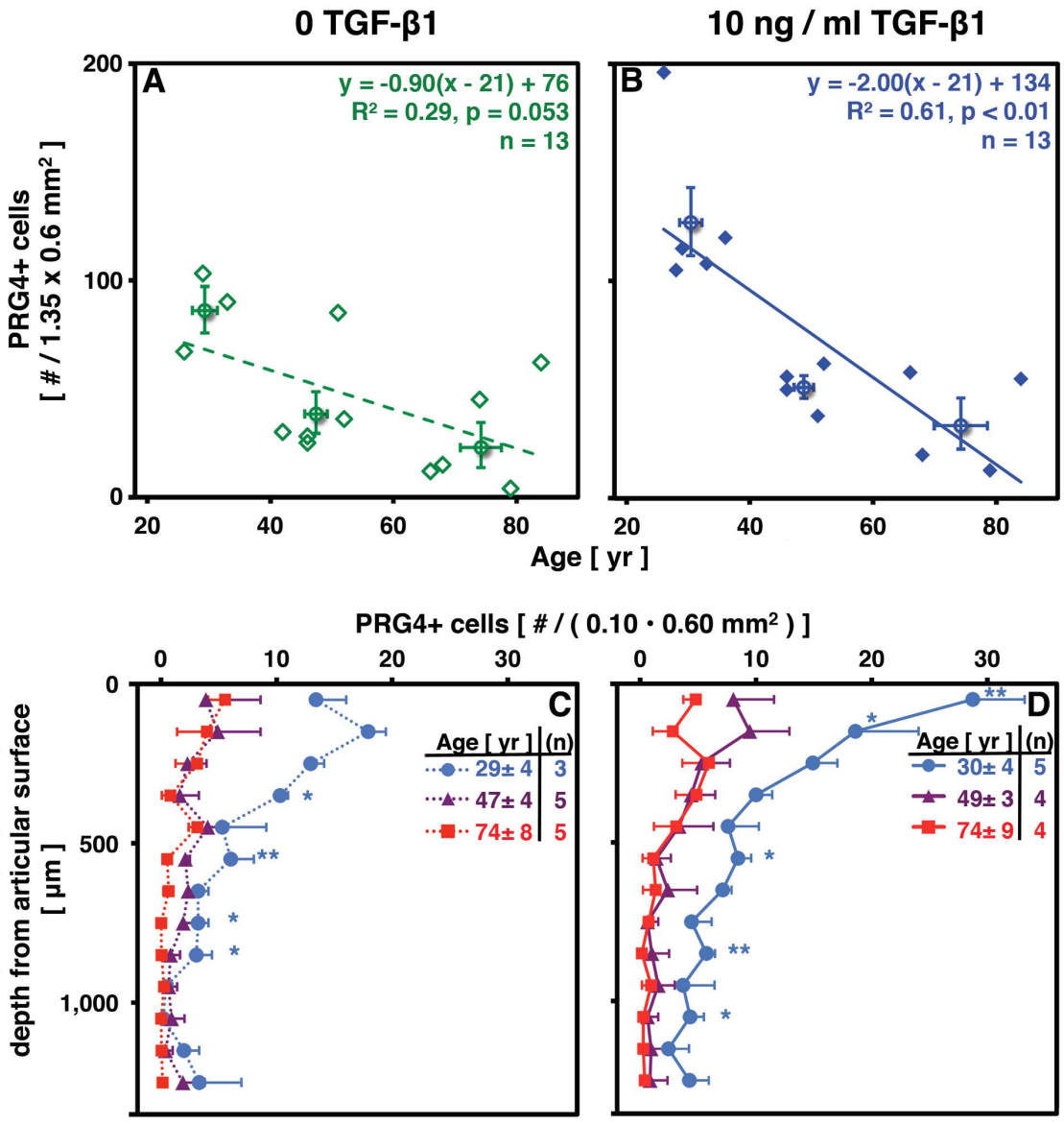


Figure 2.5: Quantification of PRG4+ cells in cultured explants of human cartilage from lateral femoral condyles. Quantification of PRG4+ cells by immunolocalization (**A,B**) in a 1.35 mm x 0.60 mm area of vertical sections and (**C,D**) as a function of depth from the articular surface in explants incubated for 6 days in medium in the (**A,C**) absence or (**B,D**) presence of 10 ng/ml TGF-β1. (**A,B**) Mean (•) and SEM (+) denoted within each age group of 20–40, 41–60, ≥61 yr. (**C,D**) PRG4+ cells as a function of depth from the articular surface in donors grouped by age (blue=20–40, purple=41–60, and red=≥61 yr). *p<0.05, **p<0.01 relative to Young group.

2.5 Discussion

This study demonstrates that, in normal human articular cartilage, constitutive and TGF-β1-stimulated *PRG4* gene expression and PRG4 protein secretion decrease with increasing age, and that these age-dependent declines are associated with decreases with age in the number of PRG4+ chondrocytes without a marked alteration in the density of chondrocytes in the superficial zone. Constitutive analysis of cartilage indicated *PRG4* mRNA (**Fig. 2.1A**) and PRG4-expressing cells (**Fig. 2.1B**) were lower in explants from older donors. Incubation with TGF-β1 of cartilage maintained PRG4 secretion at steady levels over 6 days (**Figs. 2.2B,C**), the majority of which is newly synthesized. The TGF-β1 stimulation of cartilage *PRG4* gene expression (**Figs. 2.3A,B**) and PRG4 protein secretion (**Figs. 2.3C,D**) over basal levels was blunted with increasing donor age, as was the density of PRG4-expressing cells (**Fig. 2.5B**), particularly near the articular surface (**Figs. 2.1G and 2.5D**). Collectively, these suggest that changes in activity and responsiveness of chondrocytes underlie age-related declines in PRG4 production.

A number of issues were considered in the experimental design, and affect the interpretation of the results. Site-associated heterogeneity in *PRG4* gene expression and PRG4 protein secretion exists, with variation in anterior vs. posterior regions of the condyle [8], in lateral vs. medial condyles [33], and in responses of lateral vs. medial condyles to continuous passive motion [36]. To control for such site-related variation, cartilage explants were harvested from local regions of the LFC and randomized to treatment groups. To focus on age-related rather than OA-associated

variations, the knees studied were screened to be non-OA, and only articular cartilage without macroscopic erosion was used. Such sample selection does not preclude mild histological alterations of the articular surface. Synovium [47], meniscus [48], and ligament [50] are additional sources of PRG4 and these various sources may contribute to PRG4 at the articular surface. In the analysis of PRG4 secretion based on conditioned medium, a portion may have been pre-formed PRG4 from cartilage matrix. However, particularly for TGF-β1-stimulated samples, this was estimated to be minimal based on studies with CHX. Cartilage *PRG4* gene expression and protein secretion were studied using ~400 μm explants to enable sensitive detection of age-dependent changes, since the majority of cells, total [55] and PRG4+, are near the articular surface, with some limited staining of chondrocytes in deeper regions [43], as indicated by IHC. PRG4+ cells were expressed as a function of depth from the articular surface but not normalized to total cells, as PRG4- cells were not quantified. The use of monensin (at particular concentration and duration) to amplify intracellular PRG4 signal [24, 43, 47] may influence the detection of PRG4+ chondrocytes by IHC, including the apparent depth-associated variation, but allows clear distinction of relative expression by individual cells. The depth at which chondrocytes express PRG4 can vary, approximately doubling over basal conditions to after stimulation with TGF-β1 or mechanical shear [34, 43]. The range of doses tested (0–10 ng/ml) in the present study spans the range of TGF-β1 levels in synovial fluid in health, OA, and RA [12, 38], and 10 ng/ml TGF-β1 allows comparison to previous studies of TGF-β1 responsiveness in human chondrocyte [14] and bovine cartilage explant [43] cultures. Together, the analysis of *PRG4* gene expression, protein release, and protein

localization indicate an age-associated reduction in basal and TGF-β1-stimulated cartilage PRG4 production.

The physiological bases for diminished cartilage PRG4 secretion with donor age include age-related reductions in overall cellularity, including a proportionate reduction in PRG4+ cells (**Fig. 2.6B**), a specific reduction in PRG4+ cells (**Fig. 2.6C**), as well as changes in the propensity of aged chondrocytes to secrete PRG4 (**Fig. 2.6D**). PRG4 immunolocalization, to the articular surface and in some cells of all explants, allowed analysis of the cellular changes that underlie diminished *PRG4* gene expression and PRG4 protein secretion (**Fig. 2.6**). The reduction in the number of PRG4+ cells with aging, both constitutively (**Figs. 2.1B–E**) and in response to TGF-β1 (**Figs. 2.5A,B**) may have been due to aging-related reductions in the number of cells. However, overall cell density in the superficial slices of normal human cartilage from the LFC, both overall and in the anterior region [55] does not vary markedly with age, suggesting that age-dependent decrease in cellularity, due to either an overall decrease of cells in the superficial zone (**Fig. 2.6B**) or a specific decrease in PRG4+ cells (**Fig. 2.6C**), is minor. Thus, it is likely that the age-dependent reductions in *PRG4* expression and PRG4 secretion by cartilage is due to a generally maintained number of chondrocytes, of which fewer secrete PRG4 (**Fig. 2.6D**). Consistent with this, in TGF-β1-stimulated cartilage, the age-related reduction in PRG4 secretion (–17% per decade relative to a 21 yr donor) is largely accounted for by a concomitant age-related reduction in PRG4+ cells (–15% per decade). Whether the same chondrocytes in young adults change phenotype, or represent different population of cells due to a combination of cell death and replacement, remains to be determined.

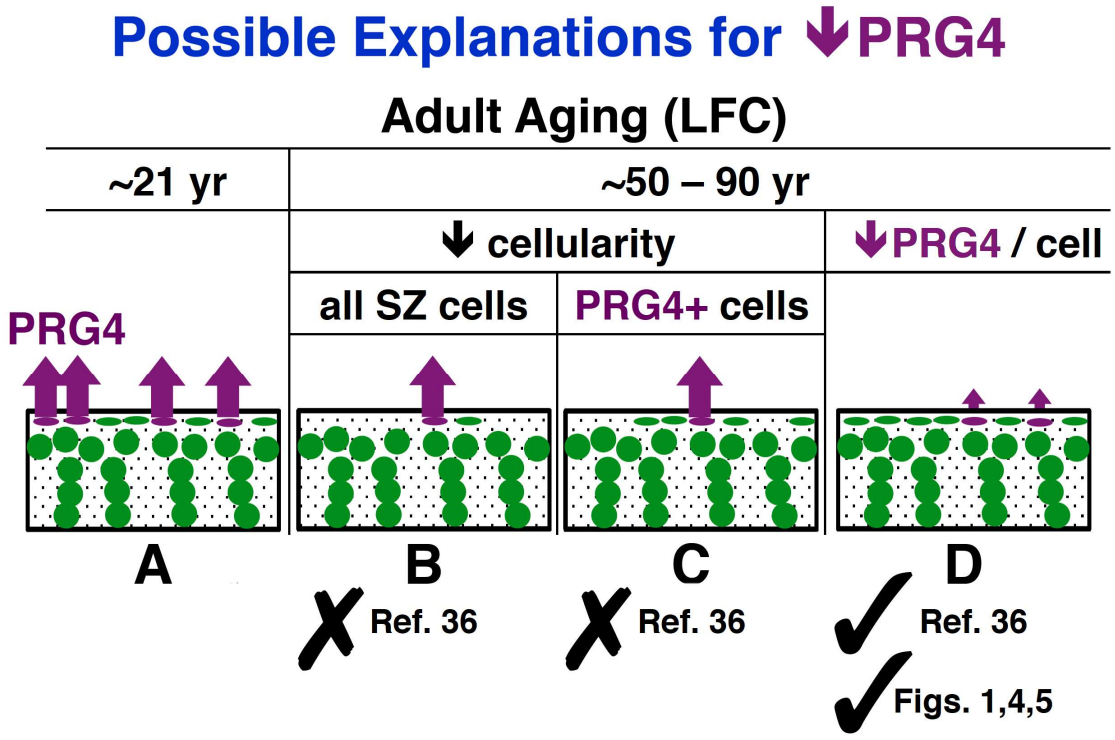


Figure 2.6: Schematic representation of possible age-related changes underlying age-dependent reduction of PRG4 secretion by human cartilage. Compared to a **(A)** young adult, possible age-related differences include (**B,C**) lower cellularity, either due to (**B**) an overall loss of cells in the superficial zone (SZ) of cartilage or (**C**) a specific loss of PRG4+ cells, or (**D**) blunted cell response, resulting in fewer cells responding as PRG4+. Under each column, evidence supporting (✓) or countering (✗) each possible explanation is shown.

The diminished constitutive and TGF-β1-stimulated gene expression and protein production in the Middle and Old age groups is consistent with the increased incidence of OA with aging in the adult. Differences between constitutive and TGF-β1 responses may reflect the complex milieu of mechanical and chemical factors, of which TGF-β1 is one, within the synovial joint. Cartilage PRG4 secretion with aging may be influenced by age-associated changes in the concentrations of these various factors, or cell responses to them, or both. The relative differences in gene expression and protein production, most notably in the marked drop in expression from Middle to Old donors, whereas secretion appears to decrease with age, may reflect pre-formed protein or alternative splice-forms of PRG4, or may be due to other reasons for poor predictive correlation between mRNA and protein [15]. The decreased stimulation by TGF-β1 of PRG4 secretion by cartilage with age (–17% per decade relative to a 21 yr donor) indicates age-related alterations in one of the major biosynthetic sources of lubricants in the joint.

The reduced PRG4 responsiveness to TGF-β1 is consistent with previous reports of age-related changes in TGF-β1 signaling and responses in human chondrocytes. With aging, chondrocyte SMAD signaling is altered, and TGF-β1 stimulation of proliferation and GAG synthesis is diminished [14, 16]. TGF-β1 signaling can occur through activin-like kinase 5 (ALK5) and activin-like kinase 1 (ALK1) receptors, with each mediated by different sets of Smad proteins and modulating different downstream effects. Age-related changes in the ALK1/ALK5 ratio, with ALK5 expression decreasing more than ALK1 [3], may also contribute to age-related changes. Age-related decreases in TGF-β1 stimulated *PRG4* expression

and PRG4 secretion may also be related to age-related loss of chromatin protein HMGB2 [53]. A number of age-related chondrocyte responses to other factors have also been reported and reviewed [27].

This study demonstrates that *PRG4* expression and PRG4 secretion by normal human cartilage from LFCs are diminished with aging, constitutively and in response to TGF-β1, as a result of a lower number and percentage of chondrocytes that express PRG4 with aging, without age-related decreases in overall cellularity. Decreased cartilage secretion of PRG4 with aging suggests an age-associated decrease in PRG4 concentrations in synovial fluid and at the articular surface of the superficial zone of cartilage. Age-associated decreases in the concentrations of PRG4 may predispose articular cartilage to increased friction and wear, which may be one factor in the development of age-associated OA. Finally, treatment strategies to stimulate joint lubrication [19, 54] may be effective for older patients, who may have an age-associated deficiency in PRG4 synthesis and secretion constitutively.

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CHAPTER 3:

NORMAL AND OSTEOARTHRITIC HUMAN SYNOVIAL FLUID REGULATION OF ARTICULAR CARTILAGE PROTEOGLYCAN 4 SECRETION: ROLE OF TGF-β, IL-1, TNF SIGNALING

3.1 Abstract

Objective: PRG4 is a mucinous glycoprotein in synovial fluid (SF) that normally lubricates articular cartilage in a dose-dependent manner, but is at variable concentrations in health and disease. A number of growth factors and cytokines regulate chondrocyte secretion of PRG4 *in vitro*. While a complex mixture of such factors is present in SF, bathing articular cartilage, those active in regulating chondrocyte PRG4 secretion are unknown. The objectives were to determine if (1) normal (NL) and osteoarthritic (OA) human SF (hSF) regulate cartilage explant secretion of PRG4, and (2) if such regulatory responses could be modulated by inhibition of TGF-β, IL-1, and TNF signaling.

Methods: Articular cartilage explants were harvested from the femoral condyles of bovine stifle joints. hSF was aspirated from knee joints of cadaveric donors and OA patients at the time of joint replacement, with IRB approval. Explants were incubated for 6 days in medium, alone as a negative control, with rhTGF-β1, rhIL-1α, or rhTNF-α as positive controls, and with one or more of the following: (1) NL-hSF, OA-hSF, or human serum (HS), (2) LY2157299 (inhibitor of TGFβ receptor I kinase signaling), (3) IRAP (blocker of IL-1 binding), (4) Etanercept (decoy receptor binding TNF). Conditioned medium was analyzed for secreted (bovine) PRG4 and nitrate.

Results: Cartilage PRG4 secretion was stimulated by OA-hSF and NL-hSF, but not HS, with the largest stimulation by 5% OA-hSF, compared to basal medium (17.8 vs. 2.6 ug/[cm^2•day]). LY2157299, IRAP, and Etanercept all substantially reversed (>84%) the marked regulation of cartilage PRG4 secretion by exogenous addition (to OA-hSF) of TGF-β1 (stimulation), IL-1α (inhibition), and TNF-α (inhibition), respectively, back near OA-hSF levels. LY2157299, IRAP, and Etanercept did not substantially modulate the regulatory effects of OA-hSF on cartilage PRG4 secretion, with LY2157299 having a small effect, and IRAP and Etanercept having no statistically significant effects.

Conclusion: hSF contains endogenous regulators of chondrocyte PRG4 secretion, causing a net stimulation that cannot be solely accounted for by activity through any of the TGF-β, IL-1, and/or TNF signaling pathways, individually.

3.2 Introduction

Proteoglycan 4 (PRG4) is a mucinous glycoprotein [40] present in synovial fluid (SF) that, under normal conditions, functions as a boundary lubricant to facilitate low friction and low wear articulation of cartilage in synovial joints [3]. PRG4 lubricates articulating cartilage in a dose-dependent manner [19, 36, 39]. With joint injury or disease, SF PRG4 concentrations are altered from normal concentrations of ~0.035–0.29 mg/ml [9, 23, 25, 34]. Low concentrations of PRG4 are associated with increased wear of cartilage after knee injury [9, 10]. Some patients with knee osteoarthritis (OA) have SF with lower than normal PRG4 concentration and boundary-lubricating function, the latter of which is restored *in vitro* upon PRG4 supplementation [23]. The concentration of PRG4 in SF is a function of a mass balance of its rates of synthesis and secretion, degradation, and/or loss from the joint, as well as changes in SF volumes [5]. One critical determinant of PRG4 concentration in SF is its rate of synthesis by chondrocytes from the superficial zone of cartilage [5, 37].

Cartilage PRG4 synthesis and secretion *in vitro* is regulated by a number of biochemical and biomechanical stimuli. TGF-β1[12, 20, 21, 35], a potent stimulus, and IL-1α [12, 20, 21, 35] and TNF-α [20, 21], potent inhibitors, are major regulators of cartilage PRG4 secretion, but other signaling molecules can also stimulate (BMP-7 [21], FGF-2 [21], IGF-I [12, 21, 35], PDGF [21], and oncostatin M [20]) or inhibit (retinoic acid [20]) PRG4 *in vitro*. Mechanical stimuli, such as compression [27],

shear [26], and continuous passive motion [28], modulate chondrocyte *PRG4* expression and PRG4 secretion.

SF interacts with articular cartilage and regulates some cartilage responses. In beagles, OA SF induced degenerative cartilage changes, assessed morphologically, and was associated with higher MMP-1 and MMP-3 chondrocyte gene expression and lower SF levels of TGF-β1, MMP-3, TIMP-1, and NO [47]. The molecular mediators of SF activity, however, are unclear and may involve SF constituents such as proteins derived from plasma, lubricants such as hyaluronan (HA) and PRG4, and endogenous cytokines and growth factors. Pro-inflammatory cytokines in SF include IL-1α, IL-1β, TNF-α, leukemia inhibitory factor, IL-6, IL-8, IL-17, and IL-18 [8, 13, 14]. Anti-inflammatory cytokines in SF include IL-4, IL-10, and IL-13. Growth factors including TGF-β1 and insulin growth factor 1 (IGF-I) are also present [6, 11, 30, 45], as are binding proteins, such as IL-1RA. With joint injury or degeneration, many of these are present at altered levels in SF [18].

The effects of chemical regulators on cellular responses can be inhibited by selective inhibitors. LY2157299 is a small molecule (369.42 MW) that inhibits TGF-β activity through the ALK5 pathway by abolishing TGFβ receptor I-mediated SMAD2 and SMAD3 phosphorylation resulting in reduced levels of active, phosphorylated SMAD [49]. Interleukin-1 receptor antagonist protein (IRAP) is a recombinant protein that inhibits IL-1 activity by competitively blocking their binding to the interleukin-1 type I and type II receptors. Etanercept is a protein (150 kDa) that inhibits TNF activity by functioning as a decoy receptor that binds to TNF.

Though a number of chemical regulators of cartilage PRG4 secretion have

been identified *in vitro*, the active regulators of cartilage PRG4 secretion endogenous to physiological SF interacting with cartilage are unknown. Thus, the hypothesis of this study was that human SF (hSF) regulates cartilage PRG4 secretion, with responses varying with SF from different joint states, and that this regulation is mediated by activity through TGF-β, IL-1, or TNF signaling. The objectives of this study were to determine if (1) cartilage PRG4 secretion is modulated by hSF from normal cadaveric (NL-hSF) and OA (OA-hSF) joints, and if such regulatory responses (2) vary between SF types and are different from that of human serum (HS) and (3) can be modulated by inhibition of TGF-β, IL-1, and TNF signaling.

3.3 Materials and Methods

3.3.1 Study Design

Cartilage explant cultures (n=4–6 disks per group per calf knee) from femoral condyles of immature (1–3 wk old) bovine stifle joints (n=24 calf knees) were incubated for 6 days in medium alone, as a negative control, or in medium with one or more of the following stimuli: 1) rhTGF-β1 (Peprotech), rhIL-1α (Peprotech), or rhTNF-α (Peprotech) as positive controls, 2) SF aspirated from normal knee joints of cadaveric donors or knee joints of patients undergoing total knee arthroplasty, or pooled HS (Gemini Bio-Products) and/or 3) LY2157299 (Selleckchem), IRAP (Amgen), or Etanercept (Amgen). Conditioned medium from days 4–6 of culture were analyzed for PRG4 or nitrite content. ***hSF/HS dose-response***. Explants from calf knees (n=10) were incubated in medium, alone, or with increasing concentrations (0.05%, 0.5%, 5%, 20%) of HS (n=4 lots of pooled serum), OA-hSF (n=5 donors), or NL-hSF (n=4 donors), to determine the effect of stimuli and dose-response on cartilage PRG4 secretion during days 4–6 of culture. ***Inhibitor dose-response***. Dose-response relationships for LY2157299, IRAP, and Etanercept were established. Explants from calf knees (n=7 for LY2157299, n=4 for IRAP and Etanercept) were incubated in medium, alone, or with 10 ng/ml rhTGF-β1 and increasing concentrations (0, 0.1, 1, 10 μM) of LY2157299, 10 ng/ml IL-1α and increasing concentrations (0, 2.5, 25, 250 ng/ml) of IRAP, or 100 ng/ml TNF-α with increasing concentrations (0, 0.25, 2.5, 25 μg/ml) Etanercept. Inhibitor reversal of TGF-β1-stimulated PRG4 secretion (for LY2157299) or IL-1α- (for IRAP) or TNF-α- (for Etanercept) stimulated nitrate release was assessed in conditioned medium during days 4–6 of culture. ***Inhibitor Validation in OA-hSF***. The efficacy of inhibitors, at selected doses,

in reversing TGF-β1-stimulated (for LY2157299), IL-1α-inhibited (for IRAP), and TNF-α-inhibited (for Etanercept) cartilage PRG4 secretion in the presence of OA-hSF (n=8 donors with n=8 knees for LY2157299, n=4 donors with n=4 knees for IRAP and Etanercept) was evaluated. Explants were incubated in medium with 5% OA-hSF, as negative control, additionally with each agonist (10 ng/ml TGF-β1, 10 ng/ml IL-1α, or 100 ng/ml TNF-α), as positive control, or with agonists and their corresponding inhibitors (1 μM LY2157299, 250 ng/ml IRAP, 25 μg/ml Etanercept for TGF-β1, IL-1α, TNF-α, respectively). ***Inhibition of TGF-β, IL-1, and TNF- signaling activity in cartilage incubated in medium alone, or with 5% OA-hSF***. The effects of 1 μM LY2157299, 250 ng/ml IRAP, and 25 μg/ml Etanercept on cartilage PRG4 secretion was assessed by incubating explants from knees (n=8) in medium alone, as negative control, with 10 ng/ml TGF-β1 as positive control, or with 1 μM LY2157299, 250 ng/ml IRAP, or 25 μg/ml Etanercept. To assess for regulation through TGF-β, IL-1, and TNF- signaling in cartilage incubated in medium with 5% OA-hSF, explants were incubated in medium alone, with 10 ng/ml TGF-β1, or in medium with 5% OA-hSF (n=10 donors with n=11 knees) only, or additionally supplemented with 1 μM LY2157299, 250 ng/ml IRAP, or 25 μg/ml Etanercept. Conditioned medium from days 4–6 of culture was assessed for PRG4.

3.3.2 Sample Selection

Bovine Knees. Stifle joints (n=24), one from each animal, from immature (1–3 wk old) bovines were obtained from an abattoir. ***Human Synovial Fluids***. hSFs were aspirated with approval of Human Subjects Committees from knee joints from cadaveric donors from tissue banks or from patients undergoing total knee arthroplasty. The former were considered SF from normal donors as they were collected within 72 hours post-mortem from joints graded as normal by morphologic

analysis of articular cartilage, essentially as described previously [17]. Briefly, donors were excluded if they had a history of knee OA or joint trauma or if osteophytes were present. Cartilage surfaces of the femoral condyles, trochlea, and tibial plateau were inspected macroscopically and graded using a modified Outerbridge scoring system [29] and the International Cartilage Repair Society knee map [1]. Each of 39 areas (3 areas in the trochlea, and 9 areas in each femoral condyle and tibial plateau) was scored individually as follows: 1: intact surface, 2: fibrillation, 3: fissuring, 4: erosion. Total knee cartilage scores, with a possible range from 39 (normal) to 156 (maximum severity), were calculated and translated into grades 0–IV (grade 0: normal when total score was 39, grade I: minimal change when total score was 40–58, grade II: mild change when total score was 59–78, grade III moderate change when total score was 79–97, and grade IV: severe change when total score was higher than 98. hSF from knee joints with grades 0–II were considered for inclusion in order to exclude OA knees. hSF aspirated from subjects undergoing total knee arthroplasty who had granted informed consent was considered to be OA-hSF. All hSFs were evaluated according to their color, clarity, viscosity, and aspirated volume. NL-hSFs were selected that were straw colored, clear, and high viscosity, which is representative of NL-hSFs. OA-hSFs were selected that were straw colored, clear, low viscosity, and >5 ml, which is a representative profile of OA-hSFs. NL-hSFs and OA-hSFs were age-matched as possible.

3.3.3 Cartilage Explant Harvest

Cartilage disks, 2 mm in diameter and containing the intact articular surface, were explanted from lateral and medial femoral condyles using sterile technique. Cartilage was scored perpendicular to the surface with a 2 mm dermal punch, then undercut with a scalpel to release disks containing the articular surface. Explants were

further cut using microtome blades and molds with defined heights to obtain final explant dimensions of 2 mm diameter x 0.5 mm thick containing the articular surface. Stratified sampling of explants from each knee was performed to control and balance the influence of region as a covariate of cartilage PRG4 secretion [28]. For each knee, explants were divided on the basis of condyle (lateral or medial femoral condyle) and location in the anterior-posterior axis, then distributed among experimental groups such that groups were balanced with respect to region.

3.3.4 Synovial Fluids

SF samples were clarified of cells and debris by centrifugation, and the resultant samples stored at -80 °C before use in experiments. Medium containing hSF were mixed together at the highest concentration of hSF needed for experiments (20% for hSF dose experiments, 5% for all else), then filtered through 0.45 μm syringe filters.

3.3.5 Tissue Culture Conditions

Cartilage disk explants were incubated for 6 days at 37 ºC and 5% CO_2 in basal medium, or medium one or more of the following stimuli at the standard concentration as well as the range indicated for dosimetry experiments: 1) 10 ng/ml rhTGF-β1, 10 ng/ml rhIL-1α, or 100 ng/ml rhTNF-α (2) 5% (0.05–20)% NL-hSF, OA-hSF, or HS, (3) 1 μM (0.01–10 μM) LY2157299, 100 ng/ml (1–100 ng/ml) IRAP, or 25 μg/ml Etanercept. Basal medium consisted of Dulbecco's Modified Eagle Medium, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution, 0.1 mM MEM non-essential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 25 μg/ml ascorbic acid, 0.01% bovine serum albumin, and 0.1% dimethyl sulfoxide. Conditioned

medium was collected, and replaced with fresh medium of the same type, every 2 days.

3.3.6 PRG4 Protein Secretion

PRG4 secreted into conditioned medium was quantitated by ELISA. Conditioned medium was serially diluted, adsorbed to ELISA plates, and reacted with monoclonal mouse antibody 3A4 (MD Bioproducts) to native bovine PRG4, horseradish peroxidase-conjugated secondary antibody, and ABTS substrate. Samples containing known amounts of PRG4 served as standards. PRG4 secretion is expressed normalized to cartilage surface area and number of days of incubation (μg/[cm^2·day]).

3.3.7 NO Release

NO released into conditioned medium was estimated by measuring the concentration of nitrite, a stable breakdown product of NO. Nitrite was assessed spectrophotometrically using Griess reaction with sodium nitrite as the standard. NO release is expressed normalized to cartilage surface are and number of days of incubation (nmol/[cm^2·day]).

3.3.8 Statistical Analysis

Summary statistics are presented as mean±SEM, with n=# of cartilage explants per experimental condition per knee, # of bovine knees, or # of hSF donors, as specified. Data that ranged over an order of magnitude were $\log_{10}$ transformed to improve normality and homoscedasticity. For each treatment type (NL-hSF, OA-hSF, HS), the effects of dose on cartilage PRG4 secretion were assessed by fitting a linear mixed-effects model for PRG4 secretion with fixed-effects (treatment dose), random-effects (hSF donors or HS lots, bovine knees), and repeated-effects (dose with subjects defined by unique combinations of knee and hSF donors or HS lots). Planned comparisons comparing treatment types at 5% were performed. The dose-dependent

effects of each inhibitor (LY2157299, IRAP, or Etanercept) on the corresponding agonist-stimulated cartilage response (LY2157299 on TGF-β1-stimulated PRG4 secretion, and IRAP and Etanercept on IL-1α- and TNF-α- stimulated NO release, respectively) were assessed by fitting linear mixed-effects models for the dependent variable (cartilage PRG4 secretion for LY2157299, NO release for IRAP and Etanercept) with fixed-effects (dose of inhibitor) and random-effects (knee). Planned comparisons were made comparing each dose of inhibitor to basal conditions and to the stimulated condition without inhibitor. The effects of inhibitors and exogenous stimuli in medium with OA-hSF were evaluated by fitting linear mixed-effects models for the dependent variable (cartilage PRG4 secretion for LY2157299, NO release for IRAP and Etanercept) with fixed-effects (treatment type with 3 levels: 5% OA-hSF, alone, +agonist, or +agonist and +inhibitor), random-effects (knee), and repeated-effects (treatment type with subjects defined by unique combinations of knees and OA-hSF donors). When fixed-effects were significant, pairwise comparisons were performed. The effects of inhibitors, in medium alone, and in OA-hSF, were assessed by fitting linear mixed-effects models for cartilage PRG4 secretion with fixed-effects (treatment type with 6 levels: medium alone or with TGF-β1, medium with 5% OA-hSF, or medium with 5% OA-hSF and LY2157299, IRAP, or Etanercept), random-effects (knee), and repeated-effects (treatment type with subjects defined by unique combinations of knees and OA-hSF donors). Planned comparisons were made comparing each condition to basal conditions and to medium with hSF. Statistical significance was set as $p<0.05$ and statistical analyses were performed using Systat 13 (Systat Software Inc.).

3.4 Results

3.4.1 OA-hSF Potently Stimulates PRG4 Secretion in a Dose-dependent Manner Compared to NL-hSF, HS

Cartilage PRG4 secretion responded differently to different types of stimuli (**Fig. 3.1**). Medium with OA-hSF stimulated cartilage PRG4 secretion in a dose-dependent manner ($p<0.01$), at statistically non-significant levels at lower concentrations ($p=0.95$ and $p=0.09$ at 0.05% and 0.5% OA-hSF in medium, respectively), and by +15.2 ($p<0.001$) and +8.9 ($p<0.01$) μg/[cm^2·day] at concentrations of 5% and 20% OA-hSF in medium, respectively, compared to basal. Neither medium with NL-hSF ($p=0.14$) nor HS ($p=0.11$) significantly stimulated cartilage PRG4 secretion in a dose-dependent manner. At concentrations of 5% in medium, OA-hSF-stimulated cartilage PRG4 secretion was +776% of basal, and higher compared to the NL-hSF-stimulated ($p<0.05$) or HS-stimulated ($p<0.01$) cartilage PRG4 secretion. At 5% in medium, the NL-hSF effect was not statistically different from HS ($p=1$).

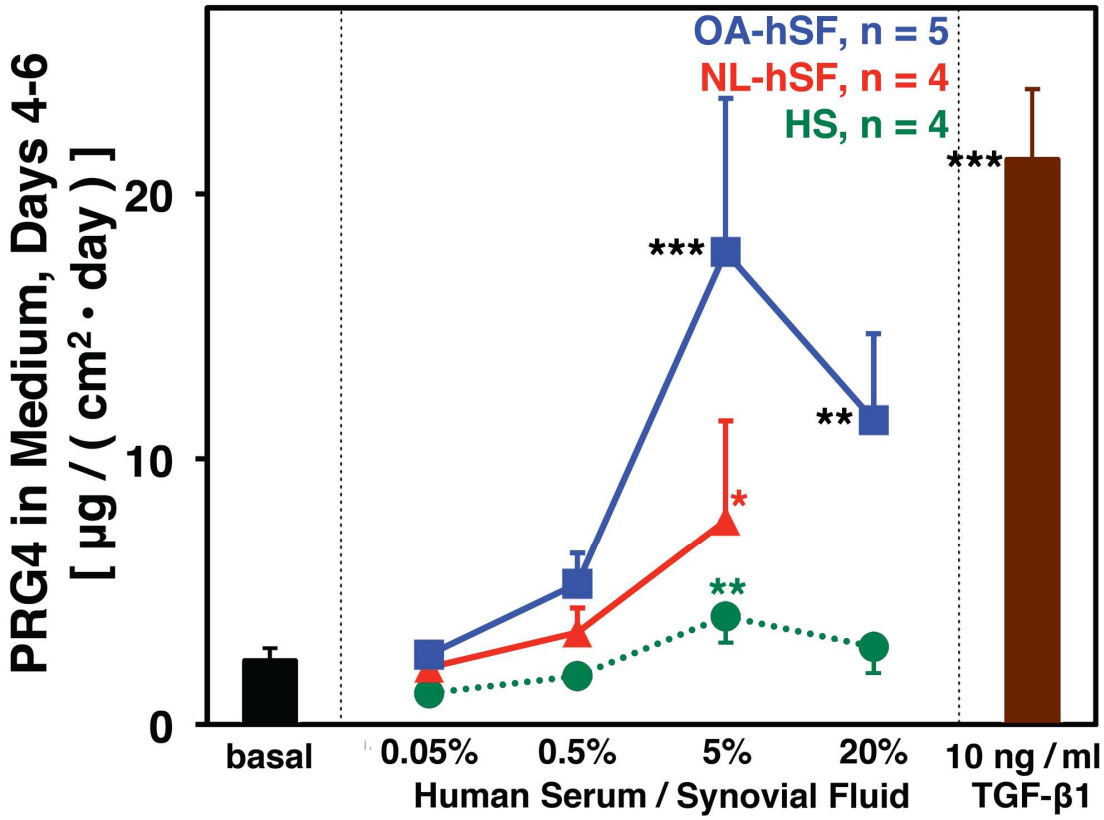

Figure 3.1: Dose-response of normal and osteoarthritis synovial fluids, and human serum, on cartilage PRG4 secretion. PRG4 in conditioned medium from days 4–6 of culture of explants (n=4–6 disks per condition per knee, total of n=10 knees) incubated in medium alone, with 10 ng/ml TGF-β1, or with increasing concentrations (0.05%, 0.5%, 5%, 20%) of hSF, either from n=4 normal (NL-hSF, in red) or n=5 osteoarthritic (OA-hSF, in blue) donors, or n=4 different lots of pooled normal human serum. Mean±SEM. *p<0.05, **p<0.01, ***p<0.001, compared to basal condition (**, *** in black, left of data points) or compared to 5% OA-hSF (* in red, ** in green, above data points).

3.4.2 Inhibitor Dose-response of Exogenous Agonist-stimulated Cartilage Responses

TGF-β1-stimulated cartilage PRG4 secretion (18.9 vs. 2.5 μg/[cm^2·day] in basal, $p<0.001$) was inhibited by LY2157299 in a dose-dependent manner ($p<0.001$), at a statistically non-significant level ($p=0.61$) at 0.1 μM of LY2157299, and by -14.9 ($p<0.001$) and -17.0 ($p<0.001$) μg/[cm^2·day] at concentrations of 1 and 10 μM of LY2157299, respectively (**Fig. 3.2A**). PRG4 secretion by cartilage incubated with 10 ng/ml TGF-β1 and LY2157299 was higher than basal at 0.1 μM LY2157299 (+15.1 μg/[cm^2·day] compared to basal, $p<0.001$), and not statistically different from basal at 1 ($p=0.13$) and 10 ($p=0.74$) μM. IL-1α-stimulated (20.7 vs. 15.2 nmol/[cm^2·day], $p=0.20$) cartilage NO release, compared to basal, was not statistically significant (**Fig. 3.2B**). IRAP did not inhibit IL-1α-stimulated NO release in a dose-dependent manner ($p=0.45$). TNF-α-stimulated cartilage NO release (42.0 vs. 15.2 nmol/[cm^2·day] in basal, $p<0.05$) was inhibited by Etanercept in a dose-dependent manner ($p<0.05$), at a statistically non-significant level at 0.25 μg/ml Etanercept ($p=0.06$), and by -28.1 ($p<0.01$) and -28.1 ($p<0.01$) nmol/[cm^2·day] at concentrations of 2.5 and 25 μg/ml Etanercept in medium, respectively (**Fig. 3.2C**). NO release by cartilage incubated with 100 ng/ml TNF-α and Etanercept was higher than basal at 0.25 μg/ml Etanercept (+9.6 nmol/[cm^2·day], $p<0.05$), and not statistically different from basal at 2.5 ($p=0.33$) and 25 ($p=0.32$) μg/ml.

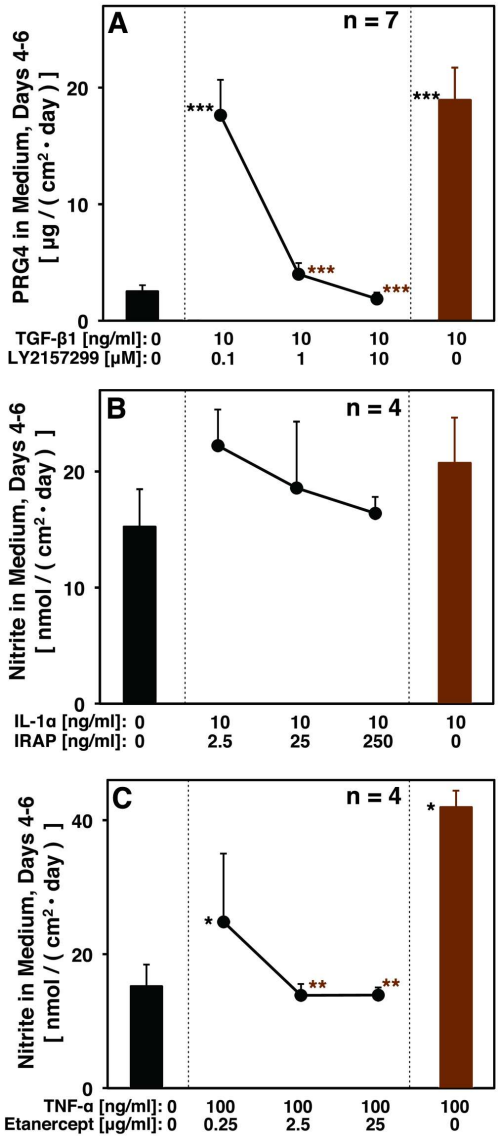

Figure 3.2: Dose-response of inhibitors on cartilage PRG4 secretion and NO release. (**A**) PRG4 in conditioned medium from days 4–6 of culture of explants from n=7 calf knees incubated in medium, alone, with TGF-β1, or with TGF-β1 and increasing concentrations of LY2157299. (**B, C**) NO in conditioned medium from days 4–6 of culture of explants from n=4 calf knees incubated in medium, alone, with (**B**) IL-1α or (**C**) TNF-α, or with stimuli and increasing concentrations of its corresponding inhibitor, (**B**) IRAP or (**C**) Etanercept, respectively. Mean±SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to basal (*, *** in black, left of data points) or compared to stimulation without inhibitor (**, *** in brown, right of data points).

3.4.3 Inhibitors Reverse Exogenous Agonist-induced Cartilage Responses in OA-hSF

LY2157299, IRAP, and Etanercept, in the presence of 5% OA-hSF, all substantially (>84%) reversed their corresponding exogenous agonist-induced cartilage responses at the doses of agonists and inhibitors indicated below (**Fig. 3.3**). In the presence of 5% OA-hSF in medium, 10 ng/ml TGF-β1-stimulated cartilage PRG4 secretion (35.7 vs. 9.1 μg/[cm^2·day], $p<0.001$) was reversed 84% by 1 μM LY2157299 (13.3 μg/[cm^2·day], $p<0.001$) to levels near 5% OA-hSF alone ($p=0.68$) (**Fig. 3.3A**). In the presence of 5% OA-hSF in medium, 10 ng/ml IL-1α-inhibited cartilage PRG4 secretion (3.5 vs. 7.2 μg/[cm^2·day] in basal, $p<0.05$) was reversed 125% by 250 ng/ml IRAP (8.2 μg/[cm^2·day], $p<0.05$) to levels near 5% OA-hSF alone ($p=0.79$) (**Fig. 3.3B**). In the presence of 5% OA-hSF in medium, 100 ng/ml TNF-α-inhibited cartilage PRG4 secretion (2.6 vs. 6.7 μg/[cm^2·day] in basal, $p<0.05$) was reversed 97% by 25 μg/ml IRAP (6.6 μg/[cm^2·day], $p<0.05$) to levels near 5% OA-hSF alone ($p=0.92$) (**Fig. 3.3C**). In the presence of 5% OA-hSF in medium, IL-1α- and TNF-α- stimulated cartilage NO release were also reversed by IRAP and Etanercept, respectively. In medium with 5% OA-hSF, 10 ng/ml IL-1α-stimulated cartilage NO release (36.4 vs. 7.2 nmol/[cm^2·day], $p<0.01$) was inhibited 92% by 250 ng/ml IRAP (9.4 nmol/[cm^2·day], $p<0.05$) to levels near 5% OA-hSF alone ($p=0.64$). In medium with 5% OA-hSF, 100 ng/ml TNF-α-stimulated cartilage NO release (25.4 vs. 7.2 nmol/[cm^2·day], $p<0.001$) was inhibited 109% by 25 μg/ml IRAP (5.6 nmol/[cm^2·day], $p<0.001$) to levels near 5% OA-hSF alone ($p=0.34$).

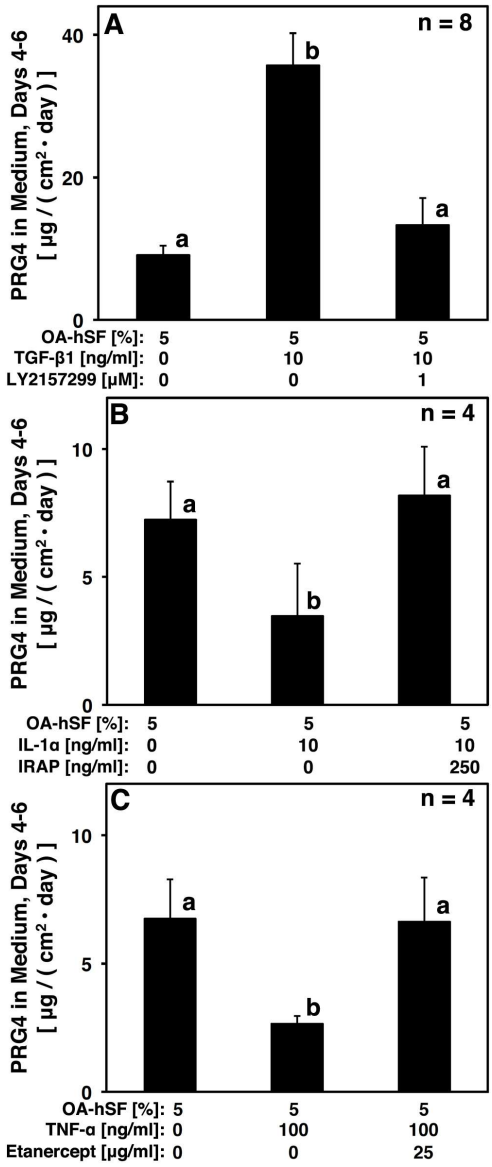

Figure 3.3: Effects of inhibitors on exogenous stimuli-induced cartilage PRG4 secretion response in medium with OA-hSF. PRG4 in conditioned medium from days 4–6 of culture of explants from (**A**) n=8 calf knees incubated in medium with OA-hSF (n=8 donors) only, with exogenous TGF-β1, or with TGF-β1 and LY2157299, or (**B, C**) n=4 calf knees incubated in medium with OA-hSF (n=4 donors) only, with exogenous (**B**) IL-1α or (**C**) TNF-α, or with stimuli and its corresponding inhibitor, (**B**) IRAP or (**C**) Etanercept, respectively. Mean±SEM. Statistical significance ($p<0.05$) of pairwise comparisons is indicated by differences in letters.

3.4.4 LY2157299, IRAP, Etanercept do not Substantially Modulate PRG4 Secretion in Medium Alone, or in Medium with OA-hSF

Cartilage PRG4 secretion was not substantially modulated by inhibitors in medium alone, without OA-hSF. PRG4 secretion by explants incubated in medium alone was markedly stimulated by TGF-β1 (+14.6 μg/[cm^2·day], $p<0.001$). The effects of inhibitors on PRG4 secretion were substantially smaller. LY2157299 (+0.9 μg/[cm^2·day], $p<0.01$) and Etanercept (1.1 μg/[cm^2·day], $p<0.01$) resulted in slightly higher PRG4 secretion, while IRAP ($p=0.51$) has a statistically non-significant effect (**Fig. 3.4A**).

OA-hSF at 5% in medium stimulated cartilage PRG4 secretion (6.3 vs. 2.1 μg/[cm^2·day] in basal, $p<0.001$). OA-hSF-stimulated cartilage PRG4 secretion was in turn modulated by LY2157299 (+1.6 μg/[cm^2·day], $p<0.05$), but not IRAP ($p=0.93$) nor Etanercept ($p=0.84$) (**Fig. 3.4B**).

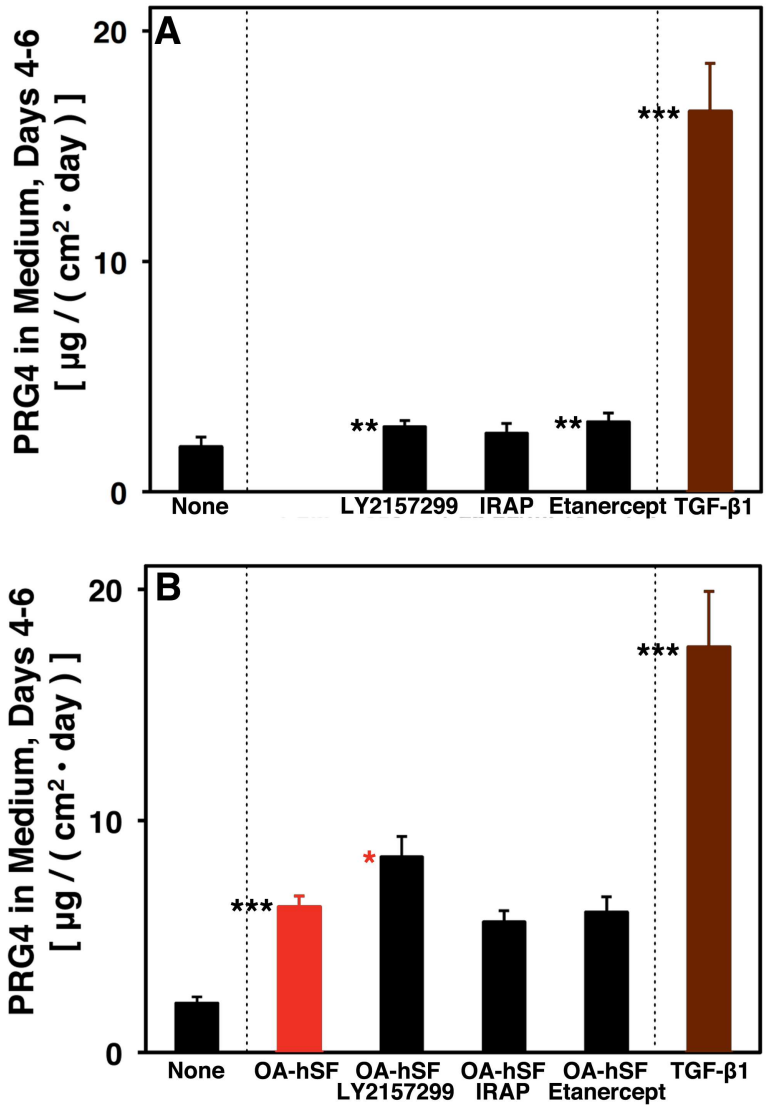

Figure 3.4: Effects on cartilage PRG4 secretion by inhibitors of TGF-β, IL-1, and TNF signaling. PRG4 in conditioned medium from days 4–6 of culture of explants (n=4–6 disks per condition per knee) incubated in medium, with 10 ng/ml TGF-β1, or with LY2157299, IRAP, or Etanercept, in (**A**) medium (with n=8 knees), or (**B**) in medium with 5% OA-hSF (n=10 donors, with n=11 knees) in the presence or absence of inhibitors. Mean±SEM. *p<0.05, **p<0.01, ***p<0.001 compared to medium alone (**, *** in black, left of data points) or compared to medium with 5% OA-hSF (* in red, left of data points).

3.5 Discussion

These findings demonstrate that hSF contains endogenous regulators that collectively result in a net stimulation of cartilage PRG4 secretion. Regulation by hSF of cartilage PRG4 secretion is dependent on dose and its disease state, and is different compared to HS (**Fig. 3.1**). LY2157299, IRAP, and Etanercept are able to block exogenous TGF-β, IL-1, and TNF activity in cartilage (**Fig. 3.2**) and in the presence of OA-hSF (**Fig. 3.3**). The effects of inhibitors on modulating OA-hSF-stimulated responses were minor, suggesting that endogenous regulators in hSF regulating cartilage PRG4 secretion cannot be attributed to any substantial effect of TGF-β, IL-1, or TNF activity alone (**Fig. 3.4**).

Several factors were considered and influenced the experimental design. Cartilage from bovine calf was used as a screening tool for assessing hSF regulation of cartilage PRG4 secretion. Some species-specific differences exist, and experiments with human cartilage would help confirm and further generalize the results to interactions between hSF and human cartilage. Since medium with hSF contains human PRG4 endogenous to hSF and also bovine PRG4 secreted by cartilage explants, ELISA quantitation of bovine PRG4 was confirmed to not cross-react with human PRG4. Since OA has a heterogeneous presentation, samples of OA-hSF were selected according to specific criteria, including color, viscosity, volume, and age of donor. In selected OA-hSF donors, samples were studied with as many as 4–6 explants each from up to 6 knees, to increase statistical power to detect donor-specific effects.

Since SF contacts and interacts with articular cartilage, these findings suggest a role for SF as an important physiological regulator of cartilage in healthy and pathological joint states. The regulatory roles of hSF may be mediated by any of the constituents that compose SF. As a plasma ultrafiltrate, SF contains many of the same proteins present in plasma [32, 33], at varying concentrations, with additional metabolic byproducts from cells lining and/or within the synovial joint. Under normal conditions, molecular size is a major determinant of the filtration properties of plasma proteins through synovial membrane and their entry into SF [7, 22, 38]; large molecular weight plasma proteins, such as fibrinogen (340 kDa), are at relatively low concentrations in SF, whereas small molecular weight plasma proteins, such as albumin (69 kDa) and transferrin (90 kDa), are at relatively high concentrations in SF [7, 22, 38]. Constituents in SF may also be predominantly synthesized by local cell populations, such as HA and PRG4. Thus, hSF is a complex biological mixture with numerous candidate regulators that may influence cartilage responses individually or in combination.

Differences in stimulation of cartilage PRG4 secretion by hSF from different joint states and human serum likely reflect differences in their compositions. Of the stimuli tested, hSF from patients undergoing total knee arthroplasty provided the most potent stimulus for cartilage PRG4 secretion. OA is associated with a number of pathological alterations in the synovial joint that perturb mass balance of SF constituents, including effusion and alterations in metabolic activity and molecular transport, leading to changes in composition of SF, and ultimately, changes in activity. Thus, differences in cartilage PRG4 secretion response to serum and SF from different

joint states are likely due to these changes in concentrations of factors, either individually or in combination. A number of biochemical factors that stimulate (TGF-β1 [12, 20, 21, 35], BMP-7 [21], FGF-2 [21], IGF-I [12, 21, 35], PDGF [21], and oncostatin M [20]) or inhibit (retinoic acid [20]) cartilage PRG4 synthesis and secretion *in vitro* are present at varying concentrations in SF in different joint states [18].

A loss-of-function approach was employed using inhibitors to block cytokine and/or growth factor activity in hSF to assess the endogenous activity of each chemical factor in regulating cartilage PRG4 secretion. TGF-β signaling [24] through binding of the ALK5 receptor and subsequent phosphorylation of SMAD proteins is a major pathway active in cartilage that can be inhibited [2, 48], though TGF-β can also signal through binding of the ALK1 receptor [15, 16] and other non-canonical signaling pathways [24, 31] in other cell types, and also in cartilage [4, 42-44]. TGF-β1-stimulated cartilage PRG4 secretion, however, appears to be primarily mediated through SMAD2-dependent pathway, as stimulation by TGF-β1 in medium is inhibited by LY2157299 (**Fig. 2A**). Since hSF is a complex biological mixture, and cartilage matrix inhibitor activity, at selected doses, was confirmed to be effective in hSF.

A number of approaches can be used to further elucidate the molecular mediators of hSF stimulation of cartilage PRG4 secretion. Inhibitor experiments suggested that IL-1, TNF, and TGF-β involvement in hSF regulation of cartilage PRG4 secretion was minor. However, interactive regulation is a possibility and may be assessed with inhibitors in combination. This study focused on the activities of IL-

1, TNF, and TGF-β since these are potent regulators *in vitro*, but the approach could be expanded to study the effects of other putative chemical regulators. Various assays to detect putative regulators in SF may be useful as a surrogate measure of activity. Since many cytokines and growth factors exist in both free and bound forms, with different activities, their activities in SF would need to be confirmed. Inhibitors of general pathways active in cartilage, such as JAK/STAT and MAPK pathways, may also be useful in helping to identify active regulators. Finally, the list of potential active regulators in hSF may be narrowed by selectively inactivating or excluding certain SF constituents on the basis of physical characteristics, such as fractionation strategies, or chemical characteristics, such as protein susceptibility to heat denaturation.

This study adds PRG4 secretion to a growing number of cartilage and chondrocyte responses that are regulated by SF [41, 46]. OA-hSF is more potent than NL-hSF in stimulating cartilage PRG4 secretion, suggesting that the regulatory environment in OA may promote PRG4 production in the joint. However, a multitude of other changes occurring with OA, such as changes within cartilage, in synovium, and in SF volume would affect mass balance of SF PRG4, necessitating further study to determine possible consequences on SF PRG4 concentrations and lubrication function.

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CHAPTER 4:

LY2109761 INTERACTS WITH HUMAN SERUM AND OSTEOARTHRITIC HUMAN SYNOVIAL FLUID TO STIMULATE ARTICULAR CARTILAGE PROTEOGLYCAN 4 SECRETION

4.1 Abstract

Objective: Proteoglycan 4 (PRG4) is a mucinous glycoprotein in synovial fluid (SF) that normally lubricates articular cartilage in a dose-dependent manner. TGF-β1 potently stimulates cartilage PRG4 secretion *in vitro* and is present in SF. The endogenous factors in SF, a complex mixture bathing cartilage, that are active in regulating chondrocyte PRG4 secretion are unknown. LY2109761 is a small molecule kinase inhibitor that blocks TGF-β signaling by inhibiting TGF-β type I and type II receptor kinase activity. The objectives were to determine if (1) LY2109761 inhibits TGF-β1-stimulated cartilage PRG4 secretion, and (2) LY2109761 modulates osteoarthritic (OA) human synovial fluid (hSF) stimulated cartilage PRG4 secretion.

Methods: Articular cartilage explants were harvested from the femoral condyles of bovine stifle joints (n=11 knees). hSF was aspirated from knee joints of

(n=10) OA patients at the time of joint replacement, with IRB approval. Explants were incubated for 6 days in medium, alone as a negative control, with rhTGF-β1 as positive control, and with one or more of the following, at the standard concentration and also the range indicated for dosimetry experiments: (1) 5% (0.05%, 0.5%, 5%) OA-hSF, or human serum (HS), and (2) 10 μM (0.1, 1, 10 μM) LY2109761. Conditioned medium during days 4–6 of culture was analyzed for secreted (bovine) PRG4.

Results: LY2109761 inhibited TGF-β1-stimulated cartilage PRG4 secretion in a dose-dependent manner, with concentrations at 1 and 10 μM LY2109761 resulting in >99% ($p<0.001$ for both) inhibition of that stimulated by 10 ng/ml TGF-β1 in medium. In medium with 5% OA-hSF, LY2109761 did not inhibit TGF-β1-stimulated cartilage PRG4 secretion. Furthermore, LY2109761 and OA-hSF interactively stimulated cartilage PRG4 secretion. This interaction effect was also present between LY2109761 and HS, and was dependent on the dose of OA-hSF or HS.

Conclusion: The effects of LY2109761 on cartilage PRG4 secretion are context-dependent, with inhibitory effects in medium with TGF-β1 and stimulatory effects in medium with OA-hSF or HS. These differences likely reflect the complex biological mixtures that are OA-hSF and HS. The interactive, stimulatory effect of LY2109761 and OA-hSF or HS on cartilage PRG4 secretion, with further study, may be a useful means of increasing cartilage production of PRG4 and joint lubrication.

4.2 Introduction

Proteoglycan 4 (PRG4) is a mucinous glycoprotein [35] present in synovial fluid (SF) that, under normal conditions, functions as a boundary lubricant to facilitate low friction and low wear articulation of cartilage in synovial joints [4]. PRG4 lubricates cartilage in a dose-dependent manner [32]. Its concentration in SF is a balance of its secretion into the joint and loss from the joint, as well as changes in SF volumes. One critical determinant of PRG4 concentration in SF is its rate of synthesis and secretion by chondrocytes from the superficial zone of cartilage [8, 33]. *In vitro*, TGF-β1[13, 17, 18, 31] is a potent stimulus for cartilage PRG4 secretion, though other chemical [13, 17, 18, 31] and mechanical [23-25] regulators exist.

TGF-β1 is present in SF [9, 26] and is secreted by some cell types in the synovial joint. *In vitro*, synovial explants, synoviocytes, and articular chondrocytes secrete TGF-β isoforms [19, 40]. It is synthesized and secreted in an inactive latent complex that is unable to bind to membrane receptors until it has been activated [2], which can occur through proteolytic cleavage, interaction with integrins, or pH changes in the local environment. Latent TGF-β ligands are synthesized in a precursor form that is non-covalently linked to latency associated peptide (LAP) and latent TGF-β binding protein [2]. In order for activation to occur TGF-β1 must be released from LAP and subsequently activated. The majority of TGF-β1 is present in the latent form (<98%, 1.84 ng/mL), while only a small fraction of the molecule is present in the biologically active form (<0.05 ng/mL) [2, 3]. Latent TGF-β1 is activated by chemical mediators, including matrix-associated metalloproteinases in articular cartilage [2].

More recently, the importance of shear force in activation has been demonstrated, with the amount of active TGF-β1 increasing from <0.05 ng/ml to 0.60 ng/ml with the application of shear forces [2].

Binding of TGF-β ligands to the extracellular domains of the type I (TβRI) and type II (TβRI) receptors initiates a sequence of signaling events. TGF-β ligand binding results in the formation of a complex of two TβRI and two TβRII receptors. The constitutively active TβRII phosphorylate TβRI receptors, which then propagate the signal through phosphorylation of SMAD2 and SMAD3 transcription factors, which then bind to SMAD4 and together translocate to the nucleus, where transcription of various target genes is regulated. Non-canonical signaling, that are SMAD-independent, also exist [12]. TGF-β signaling [21] through binding of the ALK5 receptor, a type of TβRI, and subsequent phosphorylation of SMAD proteins is a major pathway active in cartilage that can be inhibited by inhibitors [1, 41], though TGF-β can also signal through binding of the ALK1 receptor [15, 16] and other non-canonical signaling pathways [21, 27] in other cell types, and also in cartilage [5, 37-39]. TGF-β1-stimulated cartilage PRG4 secretion, however, appears to be primarily mediated through SMAD2-dependent pathway, as stimulation by TGF-β1 in medium is inhibited by LY2157299, a small molecule inhibitor of TGF-β signaling by inhibiting TβRI phosphorylation of SMAD proteins.

The downstream effects of cytokines and growth factors can be inhibited by selective inhibitors. LY2109761 is a TβRI and TβRII kinase dual inhibitor, resulting in lower levels of active phosphorylated SMAD2, that has been drawing interest as a

potential cancer therapeutic [14, 22, 42] with possible applications towards pancreatic cancer metastasis, hepatocellular carcinoma, and glioblastoma.

Thus, the hypothesis of this study was that LY2109761 inhibits TGF-β1-stimulated cartilage PRG4 secretion in medium alone and in medium with OA-hSF, and that LY2109761 modulates osteoarthritic human SF (OA-hSF)-stimulated cartilage PRG4 secretion. The aims were to determine, in bovine knee articular cartilage, if (1) TGF-β1-stimulated cartilage PRG4 secretion is inhibited by LY2109761 in medium, alone, or with OA-hSF and (2) OA-hSF-stimulated cartilage PRG4 secretion is modulated by LY2109761.

4.3 Materials and Methods

4.3.1 Study Design

Cartilage explant cultures (n=4–6 disks per group per calf knee) from femoral condyles of immature (1–3 wk old) bovine stifle joints (n=11 calf knees) were maintained for 6 days in medium alone, as a negative control, or in medium with one or more of the following stimuli: 1) rhTGF-β1 (Peprotech) as positive control, 2) SF aspirated from knee joints of patients undergoing total knee arthroplasty (OA-hSF, n=10 donors) or pooled normal adult human serum (HS, n=2 lots) and/or 3) LY2109761 (Selleckchem). Conditioned medium from days 4–6 of culture were analyzed for PRG4 content. ***LY2109761 dose-response***. Dose-response relationships for LY2109761 on TGF-β1-stimulated cartilage PRG4 secretion were established. Explants from calf knees (n=4) were incubated in medium, alone, or with 10 ng/ml rhTGF-β1 and increasing concentrations (0, 0.1, 1, 10 μM) of LY2109761, and conditioned medium during days 4–6 of culture analyzed for PRG4 content. ***Validation of LY2109761 inhibition of TGF-β1 stimulation in OA-hSF***. The efficacy of 10 μM LY2109761 in reversing TGF-β1-stimulated cartilage PRG4 secretion in the presence of 5% OA-hSF (n=4 donors) was evaluated by analyzing conditioned medium for PRG4 during days 4–6 of culture of explants (n=4–6 disks per condition, per knee, n=4 knees) incubated in medium with 5% OA-hSF, with 10 ng/ml TGF-β1 added, or with both TGF-β1 and 10 μM LY2109761 added. ***Interactive effects of LY2109761 and OA-hSF on cartilage PRG4 secretion***. The interactive effects of LY2109761 and OA-hSF on cartilage PRG4 secretion were assessed by analyzing conditioned medium during days 4–6 of culture for PRG4 from explants (n=4–6 disks per condition, per knee, n=11 knees) incubated in medium alone, or medium with 10

μM LY2109761 and/or 5% OA-hSF (n=10 donors), individually and together. ***Interaction of LY2109761 with OA-hSF or HS and dependence on concentration of OA-hSF or HS***. The dependence of the interactive effect of LY2109761 and OA-hSF on the concentration of OA-hSF was assessed by analyzing conditioned medium during days 4–6 of culture for PRG4 from explants (n=4–6 disks per condition per knee, n=2 knees) incubated in medium alone, with 10 ng/ml TGF-β1 as a positive control, or in medium with 10 μM LY2109761 and increasing concentrations (0.05%, 0.5%, 5%) of OA-hSF (n=2 donors). Interactive effects between LY2109761 and HS (n=2 lots) on cartilage PRG4 secretion, and the dependence on concentration of HS, were also assessed.

4.3.2 Sample Selection

Bovine Knees. Stifle joints (n=11), one from each animal, from immature (1–3 wk old) bovines were obtained from an abattoir. ***Human Synovial Fluids***. hSFs were aspirated with approval of Human Subjects Committees from knee joints from patients undergoing total knee arthroplasty. hSF aspirated from subjects undergoing total knee arthroplasty who had granted informed consent was considered to be OA-hSF. All hSFs were evaluated according to their color, clarity, viscosity, and aspirated volume. OA-hSFs were selected that were straw colored, clear, low viscosity, and >5 ml aspirated volume.

4.3.3 Cartilage Explant Harvest

Cartilage disks, 2 mm in diameter and containing the intact articular surface, were explanted from lateral and medial femoral condyles using sterile technique. Cartilage was scored perpendicular to the surface with a 2 mm dermal punch, then undercut with a scalpel to release disks containing the articular surface. Explants were further cut using microtome blades and molds with defined heights to obtain final

explant dimensions of 2 mm diameter x 0.5 mm thick containing the articular surface. Stratified sampling of explants from each knee was performed to control and balance the influence of region as a covariate of cartilage PRG4 secretion [25]. For each knee, explants were divided on the basis of condyle (lateral or medial femoral condyle) and location in the anterior-posterior axis, then distributed among experimental groups such that groups were balanced with respect to region.

4.3.4 Synovial Fluids

SF samples were clarified of cells and debris by centrifugation, and the resultant samples stored at -80 °C before use in experiments. Medium containing hSF were mixed together at the highest concentration of hSF needed for experiments (5%), then filtered through 0.45 μm syringe filters.

4.3.5 Tissue Culture Conditions

Cartilage disk explants were incubated for 6 days at 37 ºC and 5% CO_2 in basal medium, or medium one or more of the following stimuli at the standard concentration as well as the range indicated for dosimetry experiments: 1) 10 ng/ml rhTGF-β1 (PeproTech) (2) 5% (0.05–5)% OA-hSF or human serum (HS) (Gemini Bio-Products), (3) 10 μM (0.1–10 μM) LY2109761 (Selleckchem). Basal medium consisted of Dulbecco's Modified Eagle Medium (DMEM), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, 0.1 mM MEM Non-Essential Amino Acids Solution, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 25 μg/ml ascorbic acid, 0.01% bovine serum albumin (BSA), and 0.1% dimethyl sulfoxide (DMSO). Conditioned medium was collected, and replaced with fresh medium of the same type, every 2 days.

4.3.6 PRG4 Protein Secretion

PRG4 secreted into conditioned medium was quantitated by ELISA. Conditioned medium was serially diluted, adsorbed to ELISA plates, and reacted with monoclonal mouse antibody 3A4 (MD Bioproducts) to native bovine PRG4, horseradish peroxidase-conjugated secondary antibody, and ABTS substrate. Samples containing known amounts of PRG4 served as standards. PRG4 secretion is expressed normalized to cartilage surface area and number of days of incubation (μg/[cm^2·day]).

4.3.7 Statistical Analysis

Summary statistics are presented as mean±SEM, with n=# of cartilage explants per experimental condition per knee, # of bovine knees, or # of hSF donors, as specified. Data that ranged over an order of magnitude were $\log_{10}$ transformed to improve normality and homoscedasticity. The dose-dependent effects of LY2109761 on TGF-β1-stimulated PRG4 secretion was assessed by fitting linear mixed-effects models for PRG4 secretion with fixed-effects (dose of inhibitor) and random-effects (knee). The effects of LY2109761 in medium with OA-hSF were evaluated by fitting linear mixed-effects models for cartilage PRG4 secretion with fixed-effects (treatment type with 3 levels: 5% OA-hSF, alone, +TGF-β1, or +TGF-β1 and +LY2109761) and random-effects (knee). Pairwise comparisons between all levels were performed. The effects of interactive effects of 5% OA-hSF and 10 μM LY2109761 on cartilage PRG4 secretion were assessed by fitting linear mixed-effects models for PRG4 secretion with fixed-effects (LY2109761, 5% OA-hSF, and LY2109761*5% OA-hSF), random-effects (knee), and repeated-effects (unique combinations of knee and OA-hSF donor). The dependence of cartilage PRG4 secretion on increasing concentrations of either HS or OA-hSF in the presence of 10 μM LY2109761 was assessed by fitting linear mixed-effects models for cartilage PRG4 secretion with fixed-effects (HS or OA-hSF concentration), random-effects (knee), and repeated-

effects (unique combinations of knee and OA-hSF donor or HS lot). Significance was set as $p<0.05$ and statistical analyses were performed using Systat 13 (Systat Software Inc.).

4.4 Results

4.4.1 LY2109761 Inhibits TGF-β1-stimulated Cartilage PRG4 Secretion in a Dose-dependent Manner

TGF-β1-stimulated cartilage PRG4 secretion (21.9 vs. 2.7 μg/[cm^2·day] in basal, $p<0.001$) was inhibited by LY2109761 in a dose-dependent manner ($p<0.001$), at a statistically non-significant level ($p=0.053$) at 0.1 μM of LY2157299, and by -20.2 ($p < 0.001$) and -19.0 ($p < 0.001$) μg/[cm^2·day] at concentrations of 1 and 10 μM of LY2157299, respectively (**Fig. 4.1**). PRG4 secretion by cartilage incubated with 10 ng/ml TGF-β1 and LY2157299 was higher than basal at 0.1 μM LY2109761 (+6.7 μg/[cm^2·day] compared to basal, $p<0.001$), and not statistically different from basal at 1 ($p=0.66$) and 10 ($p=0.22$) μM.

4.4.2 LY2109761 does not Inhibit TGF-β1-stimulated Cartilage PRG4 Secretion in OA-hSF

In the presence of 5% OA-hSF in medium, 10 ng/ml TGF-β1-stimulated cartilage PRG4 secretion (46.0 vs. 12.0 μg/[cm^2·day], $p<0.01$) was not significantly inhibited by 10 μM LY2109761 (45.0 μg/[cm^2·day], $p=0.85$) and remained markedly above PRG4 secretion in conditions of 5% OA-hSF alone ($p<0.01$) (**Fig. 4.2**).

4.4.3 LY2109761 and OA-hSF Interactively Stimulate Cartilage PRG4 Secretion

Cartilage PRG4 secretion was markedly stimulated by +20.3 μg/[cm^2·day] above basal ($p<0.001$) by an interactive ($p<0.001$) effect of 10 μM LY2109761 and 5% OA-hSF (**Fig. 4.3**). Individually, 10 μM LY2109761 ($p<0.01$) and 5% OA-hSF ($p<0.001$) stimulated cartilage PRG4 secretion by +2.0 and +4.1 μg/[cm^2·day], respectively, above basal.

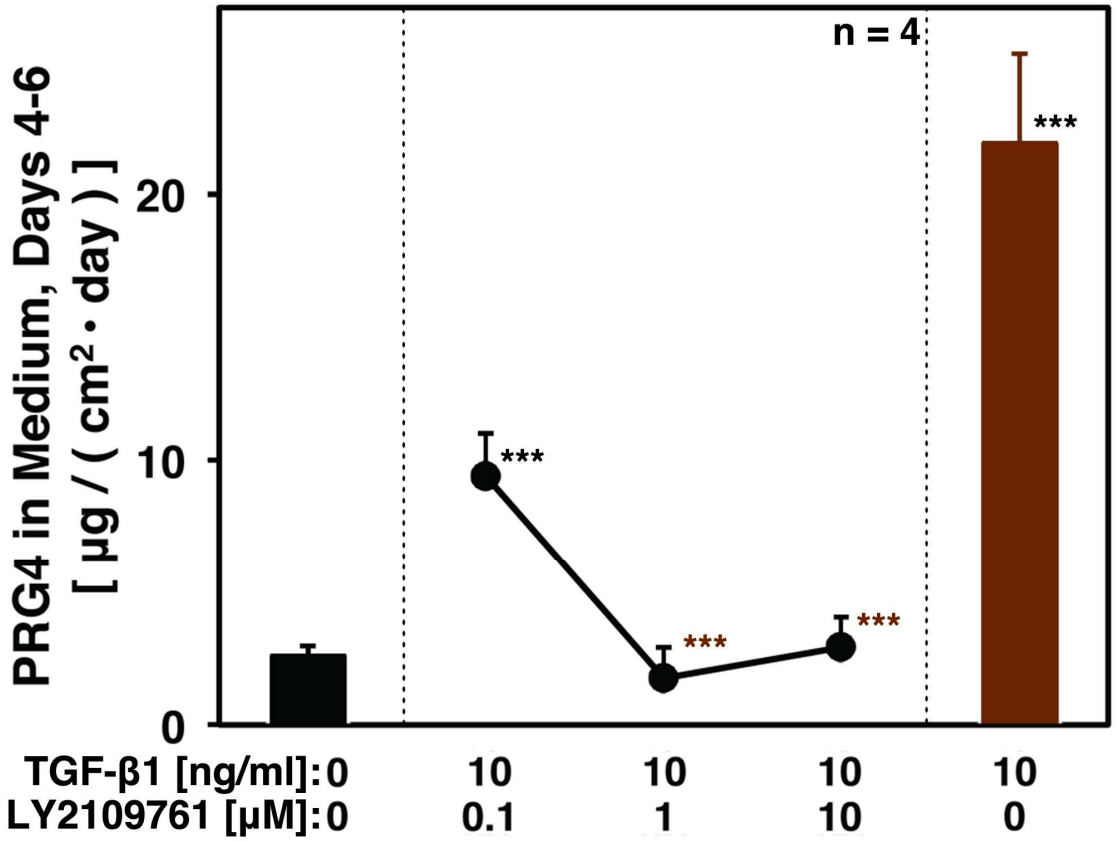

Figure 4.1: Dose-response of LY2109761 on cartilage PRG4 secretion. PRG4 in conditioned medium from days 4–6 of culture of explants from n=4 calf knees incubated in medium, alone, with TGF-β1 or with TGF-β1 and increasing concentrations of LY2109761. Mean±SEM. ***p<0.001 compared to medium alone (*** in black) or compared to medium with TGF-β1 and no inhibitor (*** in brown).

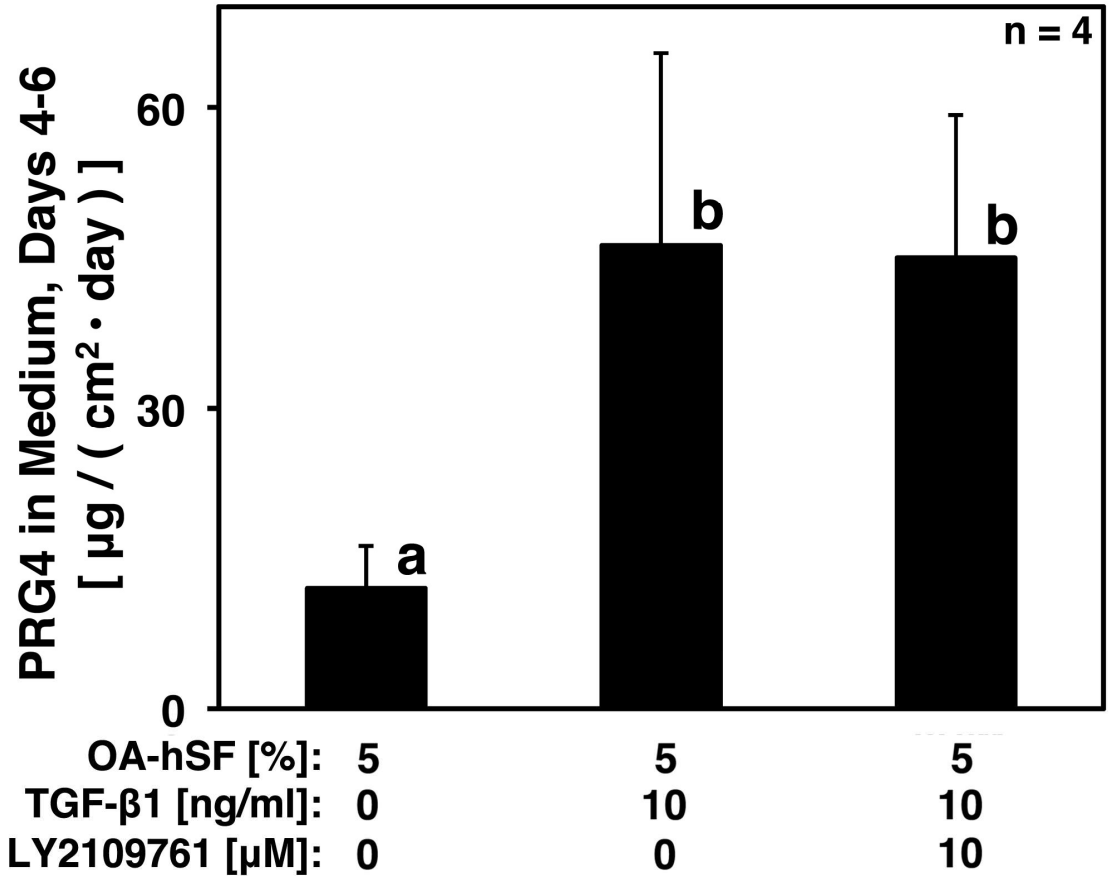

Figure 4.2: Effects of LY2109761 on TGF-β1-stimulated cartilage PRG4 secretion in medium with OA-hSF. PRG4 in conditioned medium from days 4–6 of culture of explants from n=4 calf knees incubated in medium with OA-hSF (n=4 donors) only, with exogenous TGF-β1, or with TGF-β1 and LY2109761. Mean±SEM. Statistical significance ($p<0.05$) of pairwise comparisons is indicated by differences in letters.

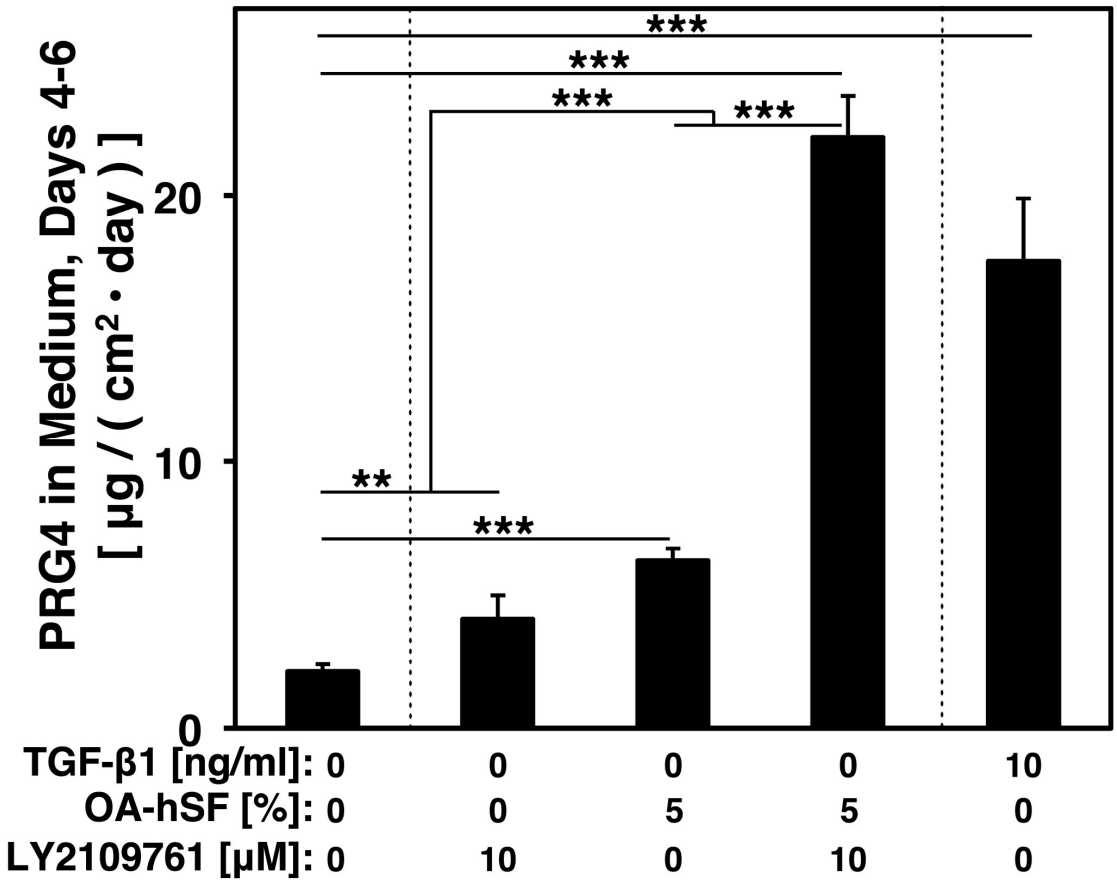

Figure 4.3: Interactive regulation of cartilage PRG4 secretion by LY2109761 and OA-hSF. PRG4 in conditioned medium from days 4–6 of culture of explants (n=4–6 disks per condition per knee, total of 11 knees) incubated in medium alone, with 10 ng/ml TGF- β1, or with OA-hSF (n=10 donors) and/or LY2109761. Mean±SEM.

4.4.4 OA-hSF and HS Dose-dependently Interact with LY2109761 and Stimulate Cartilage PRG4 Secretion

The interactive effect between 10 μM LY2109761 and either HS or OA-hSF on stimulating cartilage PRG4 secretion was dependent on the concentration ($p<0.001$) of HS or OA-hSF in medium (**Fig. 4.4**). Cartilage PRG4 secretion was markedly higher when treated with 10 μM LY2109761 in combination with 5% HS (+18.8 μg/[cm^2·day] above basal, $p<0.001$) or 5% OA-hSF (+26.4 μg/[cm^2·day], $p<0.001$).

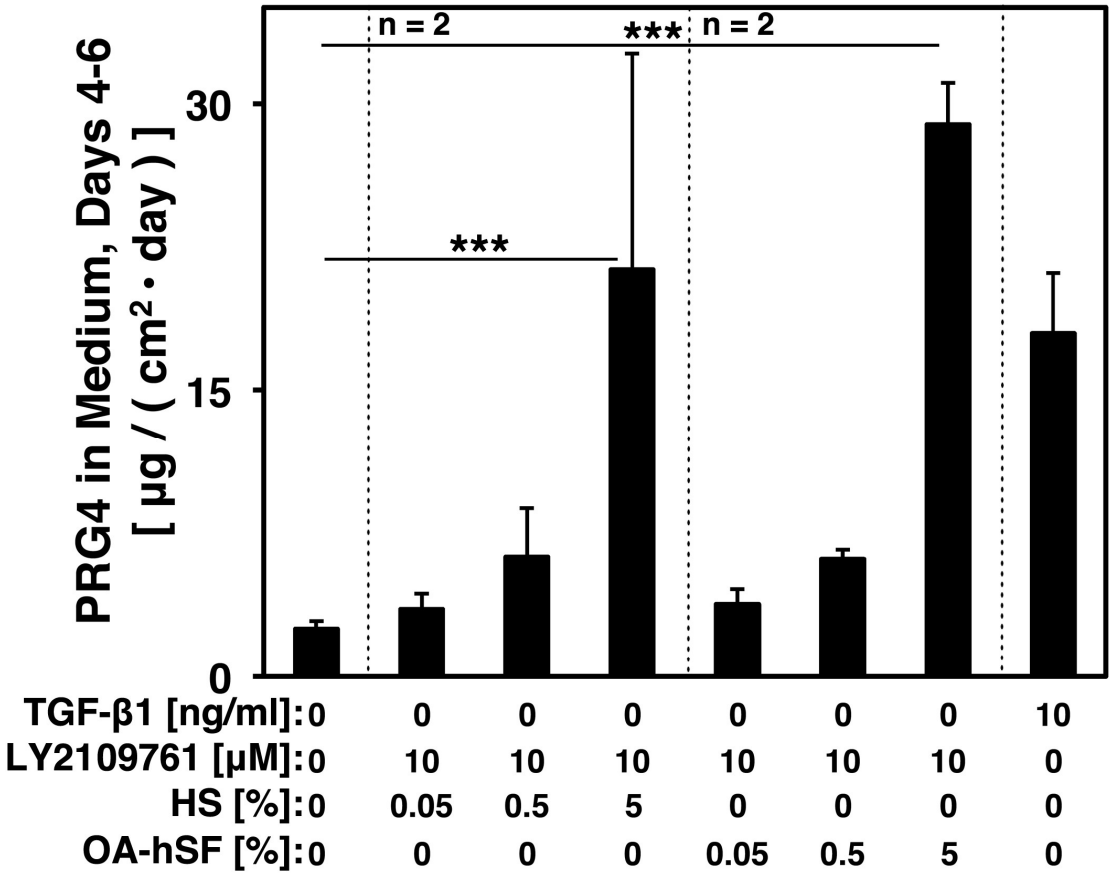

Figure 4.4: Dose-response of OA-hSF and HS on LY2109761-regulated cartilage PRG4 secretion. PRG4 in conditioned medium from days 4–6 of culture of cartilage explants (n=4–6 disks per condition) incubated in medium alone, with TGF-β1 as a positive control, and with 10 µM LY2109761 and increasing concentrations (0.05%, 0.5%, 5%) of OA-hSF from n=2 patients, or HS from n=2 lots. Mean±SEM.

4.5 Discussion

These results demonstrate that LY2109761 inhibits TGF-β1-stimulated cartilage PRG4 secretion (**Fig. 4.1**) in medium in the absence of OA-hSF or HS. LY2109761 in medium with 5% OA-hSF markedly stimulates cartilage PRG4 secretion (**Fig. 4.2**), due to an interaction effect between LY2109761 and 5% OA-hSF (**Fig. 4.3**). LY2109761 interacts with OA-hSF, and also with HS, to markedly stimulate cartilage PRG4 secretion, and the interactive effect is dependent on the concentration of OA-hSF or HS (**Fig. 4.4**).

TGF-β action is known to be different depending on the cell type and conditions. The contextual determinants that influence TGF-β action have been reviewed elsewhere [21] and include the abundance and activity of TGF-β signal transduction factors, and of transcription factors that cooperate with SMAD proteins to regulate transcription, and the epigenetic status of the cell that determines susceptibility of genes to regulation. In the presence of OA-hSF or HS, both complex biological mixtures, the altered context results in a marked stimulation of cartilage PRG4 secretion upon administration of 10 μM LY2109761. At 10 μM LY2109761, of a screen of ~80 kinases, there was >50% activity against several kinases other than TβRI and TβRII, including Sapk2a, MKK6, Lck, Yes, Fyn, and Sapk2b [22]. Though there may be some non-specific effects, LY2109761 demonstrates substantial inhibition at 1 and 10 μM of TGF-β1-stimulated cartilage PRG4 secretion when in medium alone. Whether the interactive stimulatory effect involves non-specific kinase

effects and/or altered TGF-β action due to changes in contextual determinants is unclear.

SF and HS are complex physiological mixtures containing many constituents capable of interacting with LY2109761. As a plasma ultrafiltrate, SF contains many of the same proteins present in plasma [29, 30], at varying concentrations. Under normal conditions, molecular size is a major determinant of the filtration properties of plasma proteins through synovial membrane and their entry into SF [11, 20, 34]; large molecular weight plasma proteins, such as fibrinogen (340 kDa), are at relatively low concentrations in SF, whereas small molecular weight plasma proteins, such as albumin (69 kDa) and transferrin (90 kDa), are at relatively high concentrations in SF [11, 20, 34]. Constituents in SF may also be predominantly synthesized by local cell populations, such as HA and PRG4. The interaction of LY2109761 with both HS and OA-hSF suggest that the entities mediating the interaction effect may be common to both. Thus, large molecular weight proteins, such as fibrinogen, that are at much higher concentrations in serum than in SF would seem unlikely candidates as mediators of the interaction effect. Conversely, constituents of SF that are at much higher concentrations in SF compared to serum, such as HA and PRG4, would also seem unlikely mediators of the interaction effect.

The interactive, stimulatory effect of LY2109761 and HS or OA-hSF on cartilage PRG4 secretion may be of therapeutic interest. TGF-β1 is a potent stimulus for cartilage PRG4 secretion [13, 17, 18, 31] *in vitro*, and also for collagen II synthesis *in vitro* [10, 28] and proteoglycan synthesis *in vitro* [10, 28] and *in vivo* [36]. The

involvement of aberrant TGF-β signaling in OA is suggested by reduced expression of TGF-β3 and SMAD-2P in cartilage during OA progression in two murine models of OA [7], and by reduced levels of proteoglycan synthesis and content in response to IL-1α insult when TGF-β signaling is blocked in cartilage [6]. TGF-β ligands as potential therapeutics, however, is challenging as they have pleiotropic effects and can cause side-effects such as synovial fibrosis and osteophyte formation [36]. Since TGF-β1 and LY2109761 both stimulate cartilage PRG4 secretion in the presence of OA-hSF, this suggests a possibility to counter-balance TGF-β signaling with TGF-β1 and LY2109761 while stimulating cartilage PRG4 secretion with both.

A number of approaches can be used to further clarify the nature of the interaction effect between LY2109761 and OA-hSF or HS. Since TGF-β action is known to be context-dependent, TGF-β signaling in response to LY2109761 may be altered in the presence of OA-hSF or HS compared to without, and can be assessed with techniques such as Western blot to determine the abundance of phosphorylated and total forms of the intracellular effectors of TGF-β signaling. The possible constituent(s) in hSF or OA-hSF that interact(s) with LY2109761 may be narrowed by selectively inactivating or excluding certain SF constituents on the basis of physical characteristics, such as fractionation strategies, or chemical characteristics, such as protein susceptibility to heat denaturation. Evaluating whether or not other TGF-β1-regulated responses, such as collagen II and proteoglycan synthesis, are interactively regulated by LY2109761 and medium with OA-hSF or HS would help clarify the scope of the interaction effect, if it is particular to cartilage PRG4 secretion, or if

LY2109761 in combination with OA-hSF or HS results in, paradoxically, increased TGF-β1 stimulation.

The results of this study demonstrate that the effect of LY2109761 on cartilage PRG4 secretion is context-dependent. In medium alone, LY2109761 inhibits TGF-β1-stimulated cartilage PRG4 secretion, while in medium with OA-hSF or HS, LY2109761 markedly stimulates cartilage PRG4 secretion. The interactive, stimulatory effect of LY2109761 and OA-hSF or HS on cartilage PRG4 secretion may warrant further investigation as a means of increasing cartilage production of PRG4 and joint lubrication.

4.6 Acknowledgements

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CHAPTER 5:

CONCLUSIONS

5.1 Summary of Findings

The overall objective of this work was to further the understanding of the changes in regulation of articular cartilage proteoglycan 4 (PRG4) secretion by TGF-β1 and by human synovial fluid (hSF), a complex mixture that interacts with cartilage physiologically, with aging and osteoarthritis (OA). Alterations with aging in human cartilage PRG4 secretion in response to a test stimulus, TGF-β1, was studied, in addition to possible cellular reasons underlying such age-associated changes. hSF regulation of cartilage PRG4 secretion was assessed, with hSF from both OA (OA-hSF) and NL (NL-hSF) joints evaluated and compared to human serum (HS). Inhibitors of TGF-β, IL-1, and TNF signaling were confirmed to be effective in hSF. Possible mediators of OA-hSF stimulation of cartilage PRG4 secretion were assessed with inhibitors to TGF-β, IL-1, and TNF signaling. Finally, the interactive effects of OA-hSF and a TGF-β receptor type I and II kinase dual inhibitor, LY2109761, and of HS and LY2109761, on cartilage PRG4 secretion was assessed.

PRG4 production by macroscopically normal adult human articular cartilage from the lateral femoral condyles was lower with age (**Chapter 2**), constitutively and when stimulated with TGF-β1. This reduction in cartilage PRG4 production with age coincides with reductions in the number of cells that immunostain for PRG4 (PRG4+),

without marked reduction in cellularity (**Fig. 2.6**). Constitutively, *PRG4* mRNA and the number of PRG4+ cells were lower in cartilage from older donors. Cartilage PRG4 secretion was stimulated by TGF-β1 in a dose-dependent manner, with 10 ng/ml TGF-β1 eliciting a robust PRG4 secretion over basal levels and preventing a decrease in PRG4 protein secretion that was observed in cultures of cartilage in basal medium. TGF-β1 stimulation of *PRG4* gene expression and PRG4 protein secretion both were reduced with age. Furthermore, the number of PRG4+ cells after TGF-β1 stimulation was also substantially reduced with age, without marked reduction in total cellularity.

hSF from OA joints stimulated cartilage PRG4 secretion in a dose-dependent manner (**Chapter 3**). At concentrations of 5% in medium, OA-hSF provided a significant stimulus for cartilage PRG4 secretion compared to NL-hSF and HS. LY2157299, IRAP, and Etanercept, at selected doses, all demonstrated the ability to reverse TGF-β1, IL-1α, and TNF-α induced changes to cartilage PRG4 secretion, respectively, in the presence of medium with 5% OA-hSF. OA-hSF stimulation of cartilage PRG4 secretion was not substantially modulated by any inhibitors tested, individually, with a statistically significant but small rise in cartilage PRG4 secretion with the addition of LY2157299, and statistically non-significant changes with the addition of IRAP or Etanercept, individually.

The effects of LY2109761 on cartilage PRG4 secretion were dependent on medium composition (**Chapter 4**). In medium without OA-hSF or HS, LY2109761 inhibited TGF-β1-stimulated cartilage PRG4 secretion in a dose-dependent manner, with LY2109761 at 1 and 10 μM substantially inhibiting cartilage PRG4 secretion stimulated by 10 ng/ml TGF-β1 to near basal levels. When in 5% OA-hSF or HS in medium, there was a substantial, interactive effect between LY2109761 and 5% OA-hSF or HS, markedly stimulating cartilage PRG4 secretion. The interactive effect

stimulating cartilage PRG4 secretion was dependent on the concentrations of OA-hSF or HS, with greater stimulation at higher concentrations of OA-hSF or HS.

In summary, these studies demonstrate that articular cartilage PRG4 secretion is altered with aging and degeneration and involves alterations in cartilage, hSF, and in their interactions. Changes in cartilage, involving cellular changes resulting in lower PRG4+ cells, are associated with reduced cartilage PRG4 secretion with age. The activity of hSF is also altered, with OA-hSF a more potent stimulus for cartilage PRG4 secretion compared to NL-hSF and HS, at 5% in medium.

5.2 Discussion

The major contributions of this work include advancements in the understanding of 1) the relationship between aging and cartilage PRG4 secretion and 2) the active endogenous regulators of cartilage PRG4 secretion in hSF. The clinical implications of this work are that 1) age-associated reduction in cartilage PRG4 secretion may be related to the age-associated predisposition to cartilage degeneration and OA, and thus, therapeutic strategies to restore and compensate for age-related deficits in PRG4 secretion may be useful [8, 15], 2) SF, from healthy and diseased joints, contains endogenous regulators of cartilage PRG4 secretion that may be useful targets in modulating cartilage PRG4, and 3) LY2109761 may be a small molecule of interest to increase cartilage PRG4 secretion in the presence of hSF or HS.

The finding of decreased cartilage PRG4 secretion with aging suggests an age-associated decrease in concentrations of PRG4 in SF and at the articular surface of the superficial zone of cartilage, which may predispose articular cartilage to increased friction and wear and may be one important contributor in the development of age-associated OA (**Chapter 2**). Though the associations between aging [2, 12] and PRG4 [3, 4, 11] with OA have been well recognized, the relationship between aging and PRG4 remained unclear. This work clarifies that with aging, there is an age-associated reduction in cartilage PRG4 secretion that is due to a generally maintained number of chondrocytes, of which fewer secrete PRG4, likely reflecting changes in chondrocyte phenotype, or a different population of cells due to a combination of cell death and replacement (**Fig. 2.6**). These findings are consistent with other reports of reduced

chondrocyte responses to TGF-β1 with aging [6, 7]. That cellular responsiveness, and not overall cellularity, underlies these changes suggests that early interventional strategies, prior to major decreases in cellularity that occur with progressive degeneration, targeting this aberrant response may be useful. Treatment strategies to stimulate joint lubrication [8, 15] may be effective for older patients, who may have an age-associated deficiency in PRG4 synthesis and secretion constitutively.

This work demonstrates that hSF, the complex biological mixture that contacts and interacts with cartilage physiologically, contains endogenous regulators that collectively result in a net stimulation of cartilage PRG4 secretion (**Figure 3.1**). This finding adds to the growing number of chondrocyte responses that are regulated by SF [16, 17]. Previously, though a number of chemical regulators (including TGF-β1 [5, 9, 10, 13], BMP-7 [10], FGF-2 [10], IGF-I [5, 10, 13], PDGF [10], oncostatin M [9], and retinoic acid [9]) of cartilage PRG4 secretion had been identified *in vitro*, the active regulators of cartilage PRG4 secretion endogenous to physiological SF interacting with cartilage were unknown. An approach using inhibitors to block cytokine and/or growth factor activity in hSF to assess the endogenous activity of each chemical factor in regulating cartilage PRG4 secretion was employed (**Chapters 3 and 4**). The effects of inhibitors on modulating hSF-stimulated responses were minor (**Fig. 3.4**), suggesting that endogenous regulators in hSF regulating cartilage PRG4 secretion cannot be attributed to any substantial individual effect of TGF-β, IL-1, or TNF activity alone. Since SF is a complex biological mixture with numerous candidate regulators, and contacts and interacts with articular cartilage *in vivo*, these findings suggest a role for SF as an important physiological regulator of cartilage responses in

health and pathological joint states.

This dissertation has examined several key components of the synovial joint system in aging and OA, including normal human articular cartilage from donors spanning a wide age range and hSF from normal healthy and OA joints. Taken together, the results of this dissertation have contributed to a further understanding of the alterations in regulation of articular cartilage PRG4 secretion that occur with aging and OA. These differences occur in both articular cartilage and hSF, and include a reduction with aging in the number of chondrocytes expressing PRG4, leading to diminished cartilage PRG4 secretion, and altered hSF regulation of cartilage PRG4 secretion.

5.3 Future Directions

The current work could be expanded in a number of ways. The synovial joint undergoes a number of changes with aging and OA (**Fig. 1.3**), involving cartilage, synovium, meniscus, ligaments, and SF, that would influence concentrations of PRG4 in SF and at the articular surface of cartilage. Extending the current studies of human articular cartilage and SF to include synovium, meniscus, and ligaments, and in their interactions, would enable a more complete, systems-based understanding of PRG4 homeostasis in the synovial joint. Further studies are needed to relate cartilage PRG4 secretion rates to possible consequences on SF PRG4 concentrations and, ultimately, lubrication function.

Additional studies are needed to further investigate the possible role of age-associated reduction in cartilage PRG4 secretion in contributing to the age-associated increase in OA. Though cartilage PRG4 secretion is a major determinant of PRG4 concentrations in SF, this remains to be confirmed. Early attempts at quantifying this hypothesized reduction in SF PRG4 concentrations with aging have been underpowered. The functional quality of lubrication of the PRG4 that is secreted by cartilage, and whether that varies with aging, also remains to be determined. The molecular mechanisms by which chondrocytes become less responsive to TGF-β1 signaling, resulting in a lower number of chondrocytes that express PRG4, are unclear but may be mediated by age-related changes in the ALK1/ALK5 ratio, with ALK5 expression decreasing more than ALK1 [1]. Age-related decreases in TGF-β1

stimulated *PRG4* expression and PRG4 secretion may also be related to age-related loss of chromatin protein HMGB2 [14].

The approach undertaken to evaluate endogenous TGF- β, IL-1, and TNF activity in hSF can be extended to evaluate additional candidate regulators, individually, or in combination, to elucidate the factor(s) mediating the net stimulation of cartilage PRG4 secretion by OA-hSF. Inhibitor experiments suggested that IL-1, TNF, and TGF-β involvement in hSF regulation of cartilage PRG4 secretion was minor. However, interactive regulation is a possibility and may be assessed with inhibitors in combination. The regulatory function of hSF from other joint states, such as with injury, on cartilage PRG4 secretion can be evaluated. Various assays to detect concentrations of putative regulators in SF may be useful as a surrogate measures of activity, though currently remains technically challenging given the low concentrations of many regulators in SF and the low sample volumes of hSF. Since many cytokines and growth factors exist in both free and bound forms, with different activities, their activities in SF would need to be confirmed. Inhibitors of general pathways active in cartilage, such as JAK/STAT and MAPK pathways, may also be useful in helping to identify active regulators. Finally, the regulatory function of hSF was evaluated by incubating bovine articular cartilage with hSF to facilitate the detection of cartilage secreted PRG4 without endogenous PRG4 in hSF. Experiments with human cartilage would help confirm and further generalize the results to interactions between hSF and human cartilage.

5.4 References

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