EFFECTS OF 24R,25(OH)2D3 IN THE TERATMENT OF KNEE OSTEOARTHRITIS

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EFFECTS OF 24R,25$(OH)_2D_3$ IN THE TERATMENT OF KNEE OSTEOARTHRITIS

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LIST OF ABBREVIATIONS

$1\alpha,25(OH)_2D_3$	1α,25-dihydroxy vitamin D_3
$24R,25(OH)_2D_3$	24R,25-dihydroxy vitamin D_3
ACAN	aggrecan
ANOVA	analysis of variance
cDNA	complementary deoxynuclic acid
COL2	type-II collagen
COL10	type-X collagen
COMP	cartilage oligomeric matrix protein
CSF	colony stimulating factor
DMEM	Dulbecco’s modified Eagle’s medium
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
eNOS	enzyme endothelial nitric oxide synthase
EPO	erythropoietin
FBS	fetal bovine serum
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GRO	growth regulated encogene
HGF	hepatocyte growth factor
IFN-γ	interferon-gamma
IHH	Indian hedgehog

IL	interleukin
IL-1α	interleukin 1 alpha
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
IL-7	interleukin 7
IL-10	interleukin 10
IL-12(p70)	interleukin 12
IL-13	interleukin 13
IL-17	interleukin 17
IL-18	interleukin 18
MAPK	mitogen-activated protein kinases
M-CSF	macrophage colony-stimulating factor
MCP-1	monocyte chemoattractant protein-1
MIP-1a	macrophage inflammatory protein 1 alpha
MIP-3a	macrophage inflammatory protein 3 alpha
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
OA	Osteoarthritis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDGF	platelet derived growth factor
PGE2	prostaglandin E2
PTHrP	parathyroid hormone-related peptide
RANTES	regulated on activation, normal T cell expressed and secreted
RT	reverse transcription
SDF	stem cell-derived factor
SOX9	sex determining region Y-box containing gene 9
TGF-β1	transforming growth factor, beta 1
TGF-β2	transforming growth factor, beta 2
TGF-β3	transforming growth factor, beta 3
TIMP	tissue inhibitor of metalloproteinase
TNF-a	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

CHAPTER 1

SPECIFIC AIMS

Osteoarthritis (OA) is a degenerative disease characterized by joint inflammation and cartilage degeneration due to matrix degradation and chondrocyte apoptosis. Previously, drug therapies have been developed that aim to ease pain and reduce local inflammation. Currently, no effective drug exists that has no significant side effects. Therefore, an unmet medical demand exists for development of tissue-engineering strategies to promote articular cartilage repair and regeneration to treat OA. 24R,25-dihydroxyvitamin D3 [24R,25$(OH)_2D_3$] is an attractive option for articular cartilage repair because of its anti-inflammatory and anti-apoptotic properties. 24R,25$(OH)_2D_3$, which is a naturally occurring metabolite of vitamin D_3, also has not been shown to cause toxic side effects. The *long-term goal* of this work was to develop a 24R,25-24R,25$(OH)_2D_3$ based drug therapy for articular cartilage repair and regeneration in osteoarthritis. The *objective* of the proposed research was to examine the mechanisms that cause OA and to evaluate 24R,25$(OH)_2D_3$ based therapy to promote articular cartilage regeneration and prevent disease progression. The *general hypothesis* was that 24R,25$(OH)_2D_3$ can inhibit chondrocyte apoptosis and suppress the production of catabolic factors that result in cartilage degeneration. The *overall approach* of this study used a rat *in vitro* model to study changes in OA chondrocytes and the molecular mechanisms of 24R,25$(OH)_2D_3$ in the regulation process, and *in vivo* delivery of 24R,25$(OH)_2D_3$ to repair cartilage and prevent OA progression.

<u>Specific Aim 1</u>: Determine whether 24R,25$(OH)_2D_3$ can block IL-1β induced osteoarthritic changes in an *in vitro* model.

No drug or therapy currently can effectively block the progression of osteoarthritis.

$24R,25(OH)_2D_3$ is a metabolite of vitamin D3 that has been shown previously by our lab to rescue growth plate chondrocytes from phosphate induced cell apoptosis and production of inflammatory factors that lead to cartilage destruction. Since OA is linked with joint inflammation, cell death and cartilage degeneration, we hypothesize that $24R,25(OH)_2D_3$ could potentially promote chondrocyte survival and cartilage repair to prevent the progression of OA. The purpose of the aim was to develop and characterize an *in vitro* model of OA and tests the possible therapeutic effects of $24R,25(OH)_2D_3$ on osteoarthritic chondrocytes.

Specific Aim 2: Establish an *in vivo* animal model of articular cartilage degradation and determine the effects of $24R,25(OH)_2D_3$ in preventing cartilage degeneration.

After we validate our treatments in the *in vitro* system, we established an *in vivo* animal model of cartilage degradation to further examine the effects of $24R,25(OH)_2D_3$ in preventing cartilage degeneration. The hypothesis was that increased levels of $24R,25(OH)_2D_3$ in animals with induced cartilage destruction will prevent chondrocyte apoptosis and cartilage matrix degradation. Osteoarthritis was induced in rats through anterior cruciate ligament transection (ACLT). $4x10^{-7}$ M of $24R,25(OH)_2D_3$ was delivered locally to the knee. The objective of the study was to assess effects of $24R,25(OH)_2D_3$ in combating cartilage deterioration *in vivo* by blocking inflammatory mediators and matrix metalloproteinases and to promote cartilage regeneration.

Specific Aim 3: Examine sex differences in hormone levels in human osteoarthritic joints and cell response of OA cells to vitamin D3 metabolites.

Our goal was to investigate sex differences in levels of vitamin D_3 metabolites, as well as

the difference in response of osteoarthritic chondrocytes and osteoblasts to treatment with these metabolites. It has been shown that mechanical instabilities lead to changes in the biomechanical properties of the articular cartilage, and ultimately in the biochemical properties of the cells. The hypothesis was sex differences exist that in the levels of vitamin D_3 metabolites and their receptors and in cell response to hormone treatments. Cells isolated from OA knee joints were characterized and their response to hormone treatments.

The outcomes of this study were expected to show that $24R,25(OH)_2D_3$ reduced levels of catabolic factor and induced cartilage repair that prevented osteoarthritis progression. In addition, this study provided new insights into cartilage remodeling while screening for new therapeutic agents for cartilage regeneration. The research was significant because it provided greater insight into addressing problems with current therapeutic efforts to halt, reduce joint inflammation, promote articular cartilage regeneration, and prevent the progression of OA. This insight has the potential to create better and sex specific clinical solution for articular cartilage degeneration.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

OSTEOARTHRITIS AND CURRENT TREATMENTS

Successful drug based therapy requires a comprehensive understanding of pathophysiology of the disease. Osteoarthritis is a degenerative joint disease that is characterized by the breakdown and loss of joint cartilage. Early signs for OA are collagen and aggrecan depletion in hyaline cartilage. Elevated levels of pro-inflammatory cytokines are regularly present in the synovial fluid of OA diseased joints. Collectively, these changes lead to decreased tensile and shear modulus of the cartilage and its ability to shield mechanical loads. One study has shown that the linear region shear modulus in OA decreased by 40%, from 25.5 ± 7.7 to 15.3 ± 7.2 MPa. This change can be related to the weakened collagen network and diminished contribution of cartilage during joint loading. Studies have shown that in human or large animal OA, there is a decrease in compressive stiffness and elevated permeability to fluid flow [50]. Finally, the synovium also becomes inflamed because of cartilage break down. The synovial fluid shows an increased amount of glycosaminoglycan (GAG) released from the cartilage matrix [89], which is a sign of cartilage degeneration, and less lubricin [40]. With the endogenous attempt to repair the damaged cartilage, a cascade of signaling events occur, including an increased amount of hormones and growth factors.

Impaired chondrocyte survival in the joint microenvironment contributes to the progressive degeneration of articular cartilage associated with OA [152]. Current treatment options for OA are limited. They include joint arthroscopy, cell transplantation, treatment with simple analgesics, and non-steroidal anti-inflammatory drugs (NSAIDs). While these strategies

can provide short-term pain relief, they are limited by their abilities to repair and regenerate the damaged cartilage. Eventually, patients with osteoarthritis receive a total joint replacement. Although these procedures are relatively successful in alleviating pain and restoring partial function, total joint replacements typically need implant revision surgeries every 10 to 20 years and have significantly higher morbidity and mortality with each subsequent procedure [7, 8].Therefore, therapeutic alleviation of OA has a large and unmet medical demand.

ARTICULAR CARTILAGE PHYSIOLOGY

Successful therapy of OA requires a comprehensive understanding of the articular joint's physiology. Cartilage is a flexible connective tissue found in many areas of the human bodies. Cartilage is composed of cells called chondrocytes that produce extracellular matrix (ECM) consists of collagen, proteoglycan and elastin fibers. Depending on the composition of the matrix, there are three types of cartilage in human body: hyaline cartilage, elastic cartilage and fibrocartilage. The surface of the synovial joint is covered by articular cartilage, which is a type of hyaline cartilage [9].

Articular cartilage is a highly organized tissue whose structures must remain be maintained in order to function [29]. It is organized into four zones depending on the alignment of collagen fibers. Proliferation and differentiation between these different zones is tightly orchestrated by numerous paracrine and endocrine signaling molecules. The superficial zone is the thinnest of all layers and is covered by a thin film of the synovial fluid. Water content is the highest in this zone as chondrocytes synthesize high levels of collagen and low levels of proteoglycans. Higher levels of aggrecan is found in the transitional zone, which has low cell density. In the deep zone, cells are arranged perpendicular to the surface and it contains the highest levels of proteoglycans and the lowest amount of cells. Chondrocytes in the calcified

zone express hypertrophic phenotype. The cells in this zone produce type X collagen that is responsible for providing structural support and shock absorber along with the subchondral bone. The visible border between the third and fourth zone is called tidemark and it serves as an important transition to the subchondral bone [92].

Articular cartilage provides a low-friction gliding surface and lubrication between more rigid subchondral bones around the joint. It also distributes compressive loads, which helps to reduce the peak stress on subchondral trabecular bone. The transitional zone and the deep zone are resistant to compression due to the high concentration of collagen and proteoglycan. The calcified zone acts as an anchor between the articular cartilage and subchondral bone. The tidemark in the third zone is resistant to shear. Changes in the tidemark are associated with joint diseases such as osteoarthritis [131]. In human OA patients, chondrocytes undergo terminal differentiation, from hypertrophy to mineral deposition to eventual apoptosis. Chondrocytes in OA express the marker proteins of hypertrophic chondrocytes, including alkaline phosphatase and type X collagen.

Articular cartilage is composed mainly of water (70-80% by weight), collagen type II and aggrecan. Collagen type II makes up 90-05% of the cartilage collagen and provides the tensile strength in the cartilage matrix where proteoglycan can interlace. Chondrocytes form only 1-5% of the articular cartilage volume. The major component of proteoglycan in extracellular matrix is aggrecan that provides compressive strength. Proteoglycans maintain fluid and electrolyte balance in cartilage because the subunits of proteoglycan, called glycosaminoglycans (GAGs), are negatively charged and attract only positively charged molecules that in turn bring in water to minimize the difference in osmotic pressure. Collectively, components of articular cartilage create a swollen tissue that provides resistance to compressive loading, support the distribution

of force to reduce the total force applied at cellular level and on subchondral trabecular bone to ensure tissue integrity [123, 153].

Normal and osteoarthritic articular cartilage functions can be assessed quantitatively in term of morphological properties (thickness, volume, joint surface area), and biomechanical properties (equilibrium modulus, dynamic stiffness and hydraulic permeability) as well as biological properties (composition, cell proliferation and apoptosis, protein production).

Table 2.1 Cartilage thickness

Author	Method of measurement	Mean age of donors (yrs)	Ankle-Max thickness (mm)	Ankle-mean thickness (mm)	Knee-max thickness (mm)	Knee-mean thickness (mm)
Adam et al.	Ultrasound	82.5	1.3-2.3	0.7-1.2	3.1-4.9	1.5-2.6
Shepherd and Seedhom	Needle probe	65	N/K	1.0-1.62	N/K	1.69-2.55
Sugimoto et at.	Radiographs/Persona l computer	70	1.23-2.5	0.86-1.56	N/A	N/A
Millington et al.	Hi-resolution stereo-photography	61.5	2.38±0.4	1.1±0.18	N/A	N/A

Table 2.2 Young's modulus (E)

Author	Sample		E	
Rieppo et al.	Bovine	Control	~1.1(0.2)	Properties of vitro degenerated articular cartilage
		COLL	~0.6	
		ChABC	~0.3	
		ELAST	~0.7	
Setton et al.	Canine	NOC		Properties of articular cartilage in animal models of osteoarthritis
		Post.	0.29(0.10)	
		Dist.	0.14(0.03)	
		ACLT		
		6 wk		
		Post.	0.04(0.02)	
		Dist.	0.04(0.01)	
		12wk		

		Post.	0.05(0.03)	
		Dist.	0.05(0.03)	
Saarakkala et al.	Bovine patellar	Intact	0.28(0.12)	Properties of articular cartilage during spontaneous occurring osteoarthritis
		Discolor.	0.23(0.11)	
		Superfic.	0.27(0.12)	
		Deep	0.06(0.04)	

Table 2.3 Equilibrium Shear modulus (G_{eq})

Author	Sample		G_{eq}	
Setton et al.	Canine	NOC		Properties of articular cartilage in animal models of osteoarthritis
		Post.	0.22(0.04)	
		Dist.	0.13(0.09)	
		ACLT		
		6 wk		
		Post.	0.07(0.02)	
		Dist.	0.06(0.03)	
		12wk		
		Post.	0.06(0.01)	
		Dist.	0.06(0.04)	

Table 2.4 Permeability (k)

Author	Sample		K	
Sah et al.	Rabbit	NOC	0.631(0.28)	Properties of articular cartilage in animal models of osteoarthritis
		ACLT	0.644(0.35)	
Setton et al.	Canine	NOC		
		Post.	2.4(1.3)	
		Dist.	5.0(1.7)	
		ACLT		
		6 wk		
		Post.	2.6(0.4)	
		Dist.	5.8(0.4)	
		12wk		
		Post.	4.1(1.0)	
		Dist.	6.3(1.0)	
Armstrong and Mow	Human autopsy		0.5-19.5	Properties of articular cartilage during spontaneous occurring osteoarthritis
Rivers et al.	Human	Non-OA.	4.04(2.91)	
		OA	2.92(1.00)	

NOC = non-operated controls

Pos = Positive

Post. = Posterior

Dist. = distal

ACLT = anterior cruciate ligament transection

These data suggest that in OA, not only there are changes to cartilage thickness and volume, mechanical properties of the cartilage tissue change as well. Osteoarthritic cartilage showed decreased shear and young's modulus and increased permeability.

SUBCHONDRAL BONE

Subchondral bone and articular cartilage are closely interrelated. Alteration in one can affect the structure and functional integrity of the other [52]. One of the hallmarks of OA is the pathological changes that occur in the subchondral cortical and trabecular bone. As the cartilage breaks down, the underlying bone thickens and forms bony sprouts. Significant increase in bone adsorption and remodeling occurs at the bone-cartilage interface during the early course of the disease, especially in the areas underlying the damaged cartilage. In OA joints, both subchondral cortical and trabecular bones increase early in thickness in response to the enhanced mechanical strains. With OA progression, osteoporotic changes are observed in the trabecular bone [22].

SIGNALING MOLECULES

Cartilage metabolism in most cells is a higly regulated and dynamic process. In normal

cartilage, a homsteostatic state is maintaed by chondrocytes that syntehsize new matrix molecules and degrade older molecules. This maintenance of the articular cartilage structure and function involve many sensor molecules to detect physiological changes in the cartilage and surrounding tissues and signaling pathways to maintain tissue homeostasis. As described in previous sections, the structure and function of the articular cartilage is maintained by chondrocytes that control the rate of synthesis and rate of turn-over of extracellular matrix (ECM) proteins, which contribute to tissue remodeling and repairing. The constant remodeling of cartilage tissue in response to mechanical loading is orchestrated and regulated by interaction of multiple systemic hormones and paracrine factors.

PTHrP

Studies have shown that articular chondrocyte's morphological organization is directly linked to chondrocyte function and regulation. In damaged cartilage, chondrocytes become more hypertrophic which leads to loss of its phenotype and cell apoptosis. Paracrine factors such as parathyroid hormone related proteins (PTHrP) could inhibit the terminal differentiation into hypertrophic chondrocyte. In mature articular cartilage, the primary regulatory stimulus is mechanical force, which induces the expression of PTHrP on the load bearing cartilage surface via Ca++ channel dependent integrin mechanotransduction. The PTHrP gene is tightly regulated, and its products exist at very low levels in the body. PTH/PTHrP receptors are located in the hypertrophic zone and are a sensor of PTHrP concentration in the joint region. Therefore, it senses the signal to prevent cell from further differentiation into hypertrophic chondrocytes. PTHrP is vital in maintaining cartilage mechanical function as it prevents chondrocytes from committing down the hypertrophic path, in which the secretion of main cartilage components such as aggrecan and collagen II are decreased. As it was mentioned before, collagen II and

aggrecan provide structural support for the cartilage matrix and shield stress from shear and compression loading. When there is an increase in mechanical loading, PTHrP level is up-regulated to promote chondrocyte proliferation and maintain its differentiated state to increase cartilage matrix production to distribute the extra loading. There's something very confusing here, related to PTHrP blocking differentiation but maintaining the differentiated state.

TGF-β

Articular cartilage is an avascular tissue and thus has limited intrinsic repair potential. Many growth factors have been shown to have effects on chondrocytes and cartilage repair [45]. Transforming growth factor-β (TGF-β) is one of the most important paracrine regulators of chondrocyte functions. TGF-β1, 2, and 3 have been shown to directly inhibit chondrocyte hypertrophy and promote the synthesis of cartilaginous ECM [15]. Receptors of TGF-β located on chondrocytes are a sensor of the TGF-β level due to mechanical stimulation or nutrition changes. Integrin-mediated adhesion to the ECM proteins is necessary for the optimal activation of growth factors receptors. Interaction with the integrin complex leads to partial activation of the growth factor receptor, which is then fully activated upon stimulation by its soluble ligand. TGF-β has been shown to regulate proteoglycan synthesis and contributing to chondrocyte function via binding with the two TGF-β type II receptors (TGFβRII1 and TGFβRII2) [46]. TGF-βs act on articular chondrocytes by delaying G0/G1 cells from entering the S phase and stimulate DNA replication rate and increase cell number [15]. Therefore, when there is an increase in mechanical force or increase in nutrient uptake, TGF-β expression is up regulated to promote cartilage generation to accommodate the extra mechanical loading. TGF-β mediates gene expression through SMAD transcription factors.

Interleukins

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that plays an important role in osteoarthritis pathogenesis and catabolic processes in articular cartilage [157]. IL-1β stimulates the release of catabolic factors by articular chondrocytes such as matrix metalloproteinases (MMPs) and aggrecanases that result in cartilage matrix degradation. There is also evidence that IL-1β down-regulates aggrecan and type II collagen in articular chondrocytes [157]; both are major components of the extracellular matrix in articular cartilage. IL-1β also stimulates the production of other pro-inflammatory mediators such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which lead to elevated production of prostaglandin E2 (PGE2) and nitric oxide (NO) respectively [28]. NO and PGE2 are known to be important inflammatory mediators in OA pathogenesis. NO has been shown to up-regulate the production of MMPs and pro-inflammatory cytokines such as PGE2 [42]. PGE2 mediates joint pain and regulates cartilage matrix remodeling via enhancing MMPs activity and other inflammatory cytokines [53].

MMPs and TIMPs

MMPs are a family of proteolytic enzymes, which are normally required for timely and controlled degradation of extracellular matrix components during tissue remodeling [137]. However, increased MMP expression has been closely associated with OA disease progression [116]. Among the MMPs, MMP-3 and MMP-13 play dominant roles in OA progression. MMP-13 degrades type II collagen, the main component of the articular cartilage [6]. Tissue inhibitors of MMPs (TIMPs) also play a role in articular cartilage development and osteoarthritis progression [122, 143].

24R,25-dihydroxyvitamin D_3

Studies have shown that higher serum 25-hydroxyvitamin D_3 [25(OH)D_3] levels are associated with decreased loss of articular cartilage, implying that vitamin D supplementation may prevent the progression of knee OA [35]. Vitamin D is produced upon direct exposure of the skin to sunlight or through dietary intake. It is first hydroxylated in the liver on carbon 25, producing 25(OH)D_3. This metabolite is then converted in the kidney to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] or at the 24th carbon to form 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$]. 1α,25$(OH)_2D_3$ is known to regulate bone mineralization and growth plate organization. Articular chondrocytes and subchondral osteoblasts express VDR, the genomic receptor for 1α,25$(OH)_2D_3$, suggesting a local, specific role for vitamin D metabolite mediated signaling in the joint [144]. 24R,25-dihydroxyvitamin D3 [24R,25$(OH)_2D_3$] is a vitamin D_3 metabolite that has been shown to regulate cartilage metabolism and matrix mineralization [63]. 24R,25$(OH)_2D_3$ is involved in fracture repair [127, 134]. Our studies showing that chondrocytes from the resting zone of growth plate cartilage are target cells for 24R,25$(OH)_2D_3$, indicate that it may function by promoting cartilage matrix synthesis [10, 18, 34]. 24R,25$(OH)_2D_3$ inhibits chondrocyte apoptosis induced by a variety of agents via a phosphoinositide 3-kinase-dependent pathway [18, 31]. In addition, 24R,25$(OH)_2D_3$ specifically inhibits MMP activity in cultures of chondrocytes from growth plate cartilage [34]. These observations suggest that it might block the stimulatory effects of IL-1β on chondrocyte apoptosis [165] and on these matrix degrading enzymes.

Sex Hormones

Hormones such as estrogen, testosterone, and vitamin D have been demonstrated to have direct effects on cartilage growth and the response is both sex-specific and maturation state dependent [19, 39, 76, 94, 120]. Before the age of menopause, men are more likely to develop osteoarthritis; whereas post-menopausal women are more likely to develop severe OA,

suggesting the existence of sexual dimorphism in disease development. Traditionally, estrogen has had a repressive role due to the closure of epiphyses by switching off growth hormones [70]. Estrogen and the expression of its receptor ERα are present in the nucleus and cytoplasm of articular cartilage cells and in subchondral bone, directly affecting joint metabolism [112]. 17β-estradiol (E2) was shown to inhibit doxyrubicin induced cell apoptosis and promote chondrocyte differentiation in rabbit and rat articular chondrocytes, suggesting E2 can have a protective effect on articular cartilage in OA [61, 80]. Dihydrotestosterone (DHT), the male sex hormone, was shown to reduce severity of OA in a surgical mouse model [90]. The same study also showed that ovarian hormones decreased the severity of OA in the female mice while male hormones such as testosterone, exacerbated OA in male mice. These studies suggest that sex hormones play an important role in the progression of OA.

Other Signaling Molecules

Wnts are another class of signaling molecules that play important roles in chondrogenesis. Recently, studies have suggested that Wnt signaling molecules and their inhibitor are involved in OA pathogenesis. Increased levels of β-catenin, product of canonical Wnt pathway, have been found in degenerative cartilage, suggesting Wnt signaling may contribute to cartilage loss [32]. Further investigation is needed to fully define the role of Wnt signaling in OA.

INTERACTIVE SIGNALING

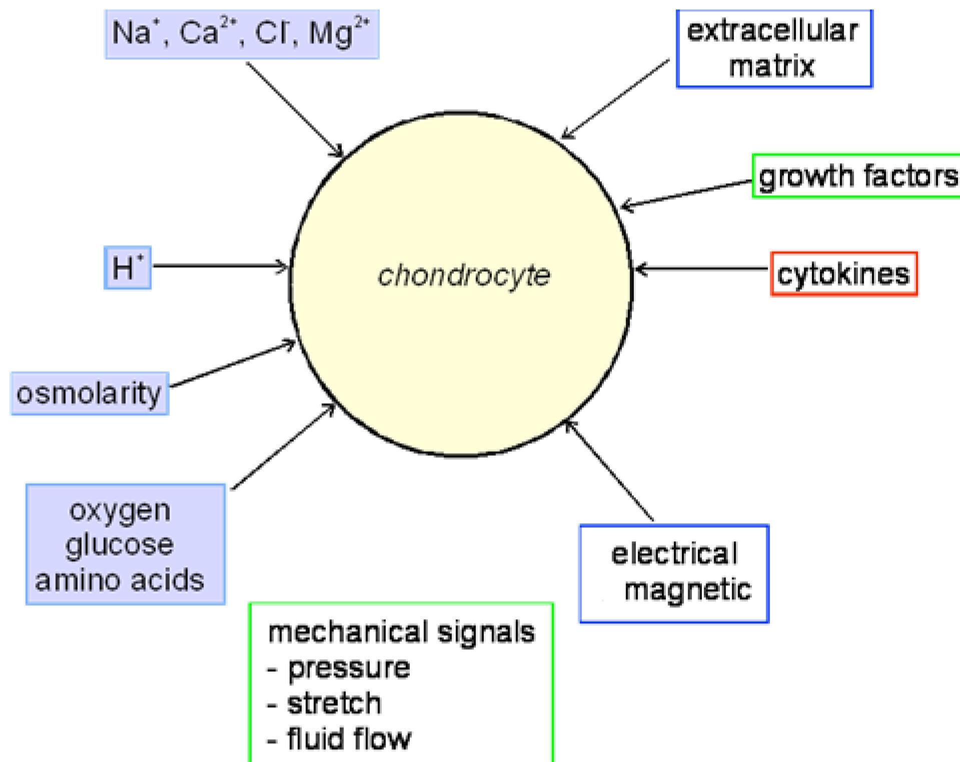

Figure 2.1 Schematic of stimuli that mediates chondrocyte fate.

Cartilage homeostasis is disrupted by mechanisms that are driven by combinations of mechanical and biological mediators including mechanical stress, injury with attendant destabilization, oxidative stress, cell-matrix interactions, and changes in growth factor responses (Figure 2.1). Chondrocytes can respond to direct biomechanical perturbation by up regulating synthetic activity or by increasing the production of inflammatory cytokines. *In vitro* mechanical loading experiments have revealed that injurious static compression stimulates proteoglycan loss, damages the collagen network, and reduces synthesis of cartilage matrix proteins, whereas dynamic compression increases matrix synthetic activity [51]. In the early stages of OA, transient increases in chondrocyte proliferation and increased metabolic activity are associated with a localized loss of proteoglycans at the cartilage surface followed by cleavage of type II

collagen. Figure 2.2 shows a schematic diagram of pathways involved in normal and OA cartilage remolding. These events result in increased water content and decreased tensile strength of the matrix as the lesion progresses.

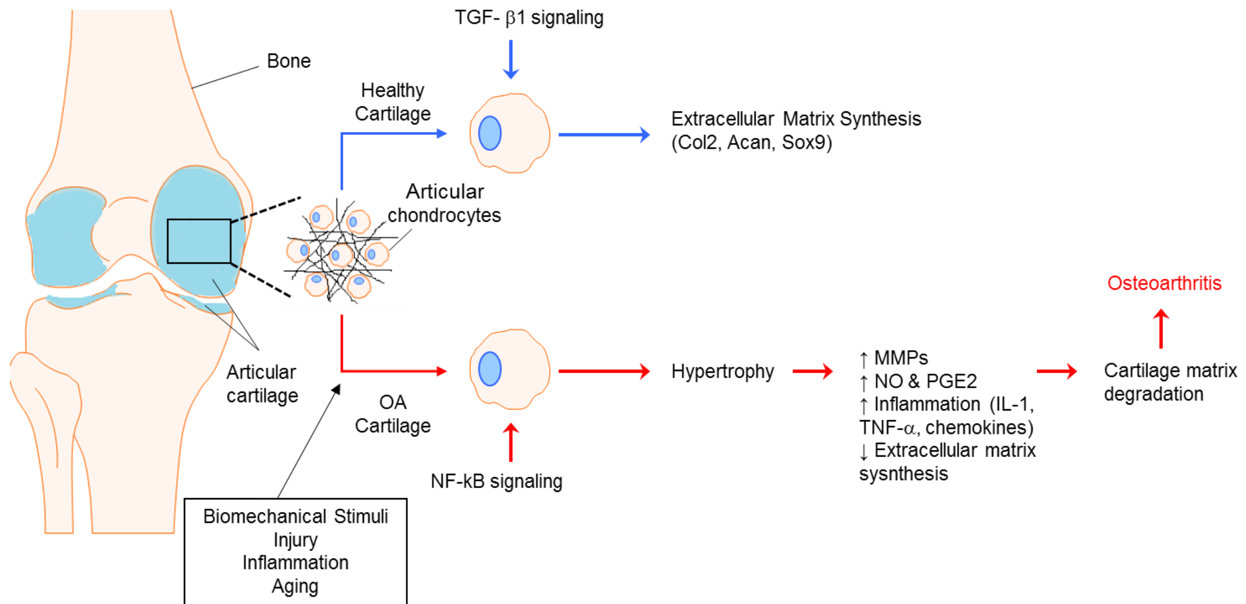

Figure 2.2 Pathways involved in normal and osteoarthritic cartilage.

Regulation of ECM depends on a highly coordinated program of chondrocyte differentiation, proliferation, and maturation involving pathways such as Indian hedgehog (IHH), PTH, TGF-β, NFkB and vitamin D_3. Studies have shown that TGF- β activates NFkB and IL-1β activates Smads [88]. PTH and TGF-β1 have shown to coregulate chondrocyte proliferation, differentiation, and matrix synthesis [101]. $1\alpha,25(OH)_2D_3$ was shown to increase TGF-beta1 protein in the chondrocyte extracellular matrix [115]. It has been suggested that with aging, chondrocytes have a decreased ability to correctly organize their ECM environment even as the correct molecules are being synthesized [7]. Thus modification of cartilage ECM expression, function, or cytokine activity could lead to alteration in cell–matrix interactions, and subsequent changes in the cellular response to mechanical stimulation and maintaining cartilage's

mechanical function.

The goal of the research on OA biology is to discover new therapeutic targets based on a better understanding of disease mechanisms. Elucidating the mechanism controlling cartilage formation will be essential in developing tissue engineering or regenerative medicine strategies for treating diseased and damaged articular cartilage.

CHAPTER 3

EFFECTS OF 24R,25(OH)2D3 IN REDUCING OSTEOARTHRITIS LIKE CHANGES IN A RAT ARTICULA CHONDROCYTES STIMULATED BY INTERLEUKIN-1β

SUMMARY

The pro-inflammatory cytokine, IL-1β has been shown to induce an osteoarthritis (OA) like phenotype in cultured chondrocytes. TGF-β1 is known to regulate chondrocyte proliferation and extracellular matrix synthesis while counteracting the deleterious effects caused by IL-1β. We have shown that 24R,25$(OH)_2D_3$ protects chondrocytes against apoptosis, suggesting that it may also block the effects of IL-1β in a manner comparable to TGF-β1. In order to test this hypothesis, an *in vitro* system that simulates OA conditions was established. Confluent cultures of rat femur articular chondrocytes were treated for 12 hours with 10 ng/ml IL-1β, followed by the addition of 10^{-7} M 24R,25$(OH)_2D_3$ or 1ng/ml rhTGF-β1 to the media. IL-1β caused dose-dependent increases in MMP-13 activity and PGE2 production. 24R,25$(OH)_2D_3$ partially reduced MMP-13 activity and PGE2 in the conditioned media compared to the IL-1β treated control cultures. This effect was similar to that of TGF-β1. In conclusion, our results show that 24R,25$(OH)_2D_3$ has an inhibitory effect against IL-1β induced increase in activity and production of catabolic factors in a rat articular chondrocyte *in vitro* culture system, suggesting 24R,25$(OH)_2D_3$ could be used as a local treatment for osteoarthritis to combat IL-1β caused deleterious changes.

INTRODUCTION

Osteoarthritis is a disease characterized by pain and limited joint mobility, affects nearly 30 million people in the United States, with current medical costs approaching and costs nearly $60 billion per year to treat [82]. Characteristics of OA include articular cartilage breakdown, subchondral bone sclerosis, and synovial tissue inflammation. Although the exact sequence of OA pathogenesis remains unclear, studies have linked inflammation, even during the early stages of disease development, with disease progression. Secreted inflammatory molecules, such as pro-inflammatory cytokines are among the critical mediators in OA pathophysiology [49]. Impaired chondrocyte survival in the joint microenvironment contributes to the progressive degradation of articular cartilage associated with OA [92, 152]. Inflammation plays an important role in osteoarthritis (OA) pathogenesis.

Current treatment options for OA are limited [64, 97, 121, 164]. Drug therapies have been developed that aim to ease pain and reduce local inflammation [139], however, no effective drug exists. Current treatments also have side effects including intestinal bleeding. Therefore, compounds that indirectly reduce inflammation may alleviate disease symptoms without significant side effects [74, 78].

Transforming growth factor beta-1 (TGF-β1) has also been shown to promote chondrocyte proliferation and to maintain chondrocytes in non-hypertrophic state [161]. Importantly, it inhibits effects of IL-1β associated with OA, and also modulates the production of $24R,25(OH)_2D_3$ [1, 55, 63, 117, 126, 148]. While TGF-β1 is chondroprotective, its value as a therapeutic agent is limited by the potential for fibrosis [149]. In contrast, $24R,25(OH)_2D_3$ is normally present in blood and is not associated with pathology. Therefore, we compared the effects of $24R,25(OH)_2D_3$ on IL-1β induced changes in articular chondrocytes to those of TGF-

β1 in order to examine the therapeutic potential of the secosteroid for treatment of OA.

As OA is linked to joint inflammation, cell death, and cartilage degeneration, we hypothesize that 24R,25$(OH)_2D_3$ could potentially promote chondrocyte survival and cartilage repair to prevent OA progression. The purpose of this study is to investigate the inhibitory effects of 24R,25$(OH)_2D_3$ on IL-1β stimulated OA-like phenotype changes in articular chondrocytes and its potential interactions with TGF-β1. In our study, we took chondrocytes from male and female human knee joints and treated them with physiological concentration of 24R,25$(OH)_2D_3$ to examine the therapeutic effect of treatment on osteoarthritic chondrocytes. Cartilage was isolated from areas of minimal-fibrillation as a control, and from areas of maximum erosion for both male and female cells. Levels of 24R,25$(OH)_2D_3$ in the synovial fluid were measured.

MATERIALS AND METHODS

Reagents

Rat IL-1β was purchased from PeproTech (Rocky Hill, NJ). 24R,25$(OH)_2D_3$ was obtained from Enzo Lifesciences (Farmingdale, NY). TGF-β1 was purchased from R&D systems (Minneapolis, MN). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless specified.

Cell Culture and rArC Phenotype Characterization

Articular cartilage was obtained from the femurs of 100-125 gram male Sprague-Dawley rats. Cartilage specimens were sliced and incubated in 0.25% trypsin for 30 minutes at 37°C. Rat articular chondrocytes (rArCs) were extracted by incubating the cartilage fragments for 16 hours in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher, Waltham, MA), 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA), and 0.03% collagenase type II

(Worthington Biosciences, Lakewood, NJ). This solution was passed through 10 μm cell strainers to remove tissue debris. Cells were cultured in 75 cm^2 flasks in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin–streptomycin (Life Technologies, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were expanded in culture until passage 4. At end of each passage, RNA was extracted and gene expressions for chondrocyte genes were determined using Real-Time PCR. Passage 1 cells were chosen for consequential experiments as it has high levels of chondrocyte genes. Passage 1 cells were plated for experiments at a density of 15,000 cells/cm^2 and media were changed 24 hours after plating and every 48 hours thereafter until cells reached confluence.

Human Tissue Isolation

Knee joints were obtained from 6 male and 6 female non-Hispanic white subjects between the ages of 65 and 75 years undergoing total joint replacement surgery at Mayo Clinic. Tissues were collected under Institutional Review Board approval by Mayo Clinic, the Georgia Institute of Technology, and Virginia Commonwealth University. Patients with a BMI >30, inflammatory arthritis, or who had a history of knee infections were excluded from the study. Cartilage pieces were isolated in aseptic conditions from minimally fibrillated and maximally eroded areas of the joint.

Experimental Design

Rat IL-1β was reconstituted in sterile PBS containing 0.1% bovine serum albumin to a stock concentration of 100 μg/ml. The stock was diluted to final concentrations in culture medium. To induce OA-like phenotype changes, confluent cell cultures were treated with 1, 5, or 10 ng/ml IL-1β for 24 hours. Caspase-3 activity and DNA fragmentation of cell lysates were determined. Nitric oxide (NO), prostaglandin E2 (PGE_2), and matrix metalloproteinase-13

(MMP-13) productions in conditioned media were measured by ELISA.

In order to determine which time point would be used to examine the effect of 24R,25$(OH)_2D_3$. We treated P1 rArCs with different combinations of time points for adding 24R,25$(OH)_2D_3$. In group one, we added 24R,25$(OH)_2D_3$ for the entire 24 hours. In group two, we only added 24R,25$(OH)_2D_3$ for the last 12 hours of the treatment. Our results show 24R,25$(OH)_2D_3$ exhibited the best inhibitory effect when it was only added at the last 12 hours of the treatment. Therefore, all the future experiments were done by adding 24R,25$(OH)_2D_3$ treatment in the last 12 hours.

To examine the effect of 24R,25$(OH)_2D_3$ on IL-1β stimulated chondrocytes, confluent cultures of rArCs were treated with 10 ng/ml IL-1β (PeproTech) for 12 hours. For the next 12 hours, cells were treated with 10 ng/ml of IL-1β or 10 ng/ml of IL-1β with 10^{-9}-10^{-7} M of 24R,25$(OH)_2D_3$. Chondrocyte gene expression was determined using RealTime PCR and levels of NO, PGE_2, and MMP-13 were measured by ELISA.

To examine the protective effect of TGF-β1 on chondrocytes stimulated by IL-1β, confluent cultures of rArCs were treated with 10 ng/ml IL-1β for 12 hours. For the next 12 hours, cells were treated with 10 ng/ml IL-1β or 10 ng/ml IL-1β with 0.1, 1, or 10 ng/ml TGF-β1. Levels of NO, PGE_2, and MMP-13 were measured by ELISA.

To investigate the interaction effect of 24R,25$(OH)_2D_3$ and TGF-β1 on osteoarthritis marker MMP-13, confluent rArCs were treated with 10^{-8} M 24R,25$(OH)_2D_3$ ± 0.1 ng/ml TGF-β1 for the last 12 hours in addition to the 10 ng/ml IL-1β treatment.

24R,25$(OH)_2D_3$'s effect on regulating TGF-β1 production and signaling was examined by treat ing confluent rArCs with 10^{-9}-10^{-7} M 24R,25$(OH)_2D_3$. Active and latent levels of TGF-β1 were determined via ELISA (RD Systems, Minneapolis, MN). mRNA levels of TGF-β1

receptor TgfbrII, signaling molecules Smad2 and Smad3 were measured using RealTime PCR.

To examine if the effect of 24R,25$(OH)_2D_3$ on MMP-13 levels is mediated through the TGF-β1, confluent rArCs were treated with IL-1β for 12 hours and prior to the addition of 24R,25$(OH)_2D_3$, antibody for blocking TGF-β1 receptor II (TgfbrII) or soluble receptor TgfbrII (RD Systems, Minneapolis, MN) was added to the media for 30 minutes. To investigate if the inhibitory effect of 24R,25$(OH)_2D_3$ on MMP-13 production is mediated through the TGF-β1signaling pathway, confluent rArCs were treated with IL-1β for 12 hours and prior to the addition of 24R,25$(OH)_2D_3$, antibody for blocking TgfbrII or soluble TgfbrII (RD Systems, Minneapolis, MN)was added to the media for two hours.

Gene Expression

RNA harvested using a TRIzol® (Invitrogen, Carlsbad, CA) extraction method following the manufacturer's protocol. mRNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). RNA (250 ng) was amplified using reverse transcription (High Capacity cDNA Reverse Transcription kit, Life Technologies). Starting quantities of mRNA were determined using SybrGreen chemistry (Power SYBR® Green PCR Master Mix, Life Technologies) in a StepOne Plus imaging system (Applied Biosystems). mRNA levels for chondrocyte genes aggrecan (Acan), type II collagen (Col2), and sex determining region Y-box 9 (Sox9), as well as for Smad2, Smad3, and receptor TGF-β1 receptor II (TgfbrII) were measured by real-time quantative PCR (qPCR). All mRNA are presented as normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

DNA fragmentation

At 90% confluence, cells were incubated with 1 μCi/ml ^{3}H-thymidine (Perkin Elmer, Waltham, MA) for four hours prior to IL-1β treatment. Cells were then treated for 24 hours with

10 ng/ml IL-1β. At the end of the treatment period, cell monolayers were lysed [10mM Tris-HCl, 1mM EDTA, 0.2% Triton X-100] and subjected to three freeze-thaw cycles. Intact DNA was separated from fragmented DNA by ultracentrifugation at 13,000*g* for 15 minutes. Intact DNA (pellet) and fragmented DNA (supernatant) were counted by liquid scintillation counting. Results are presented as percent fragmented DNA/total DNA.

Caspase-3 activity

Caspase-3 activity was determined using a colorimetric assay (CaspACE® Assay, Promega, Madison, WI). Monolayers were lysed in cold lysis buffer for 10 minutes at 4°C, and the cell lysates centrifuged at 10,000g for 1 minute. The resulting supernatant was combined with 2x reaction buffer and DEVD-pNA substrate and incubated at 37°C for 2 hours. Absorbance at 405 nm was determined using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). Caspase-3 activity was normalized to total protein content measured (Pierce 660nm protein assay from Thermo Scientific, Waltham, MA USA).

Nitric Oxide

Nitric oxide production from conditioned media from cells was measured using a 2, 3-diaminonaphthalene (DAN) fluorescent assay by measuring the total amount of nitrite and nitrate in the media. NO production was normalized to total DNA measured (Promega, Madison, WI).

Prostaglandin E2

PGE_2 production was measured in conditioned media using a competitive enzyme immunoassay (R&D Systems, Minneapolis, MN), and normalized to total DNA measured (Promega, Madison, WI).

Matrix Metalloproteinase-13

MMP-13 activity from conditioned media was determined using a fluorometric assay kit

(AnaSpec, Fremont, CA). To determine MMP-13 activity, a monoclonal anti-human-anti-MMP13 was used to pull down both pro- and active forms of MMP-13. The activity of MMP-13 was quantified by a 5-FAM/OXL 520 fluorescence resonance energy transfer (FRET) peptide and normalized to total DNA measured (Promega, Madison, WI). This assay can detect the activity of sub-nanogram of human MMP-13 without cross-reactions with other human MMPs.

Statistical Analysis

Data are presented as mean ± SEM of n=6 independent cultures per variable. All experiments were repeated to validate the results. Data presented are from one representative experiment of two trials. Data were first examined by analysis of variance (ANOVA) and post hoc test using Bonferroni's modification of Student's t-test. $P<0.05$ was considered to be significant. The value of each sample from the treated group was divided by the mean of the control group. Each data point represents the mean ± SEM for six normalized values and the control is represented by a dashed line with a value equal to one. Significance was determined by Mann Whitney test. $p \leq 0.05$ was considered to be significant.

RESULTS

The results of the rArC characterization show that P1 chondrocytes have the highest mRNA levels of Acan, Col II, Comp, type X collagen and Sox 9. P1 cells also have the lowest levels of fibrocartilage type I collagen (Figure 3.1A, 3.1B, 3.1C, 3.1D, 3.1E and 3.1F). mRNA levels of cartilage-related genes significantly decreased after passage 2, and maintained these low levels through passage 4. Therefore, for our future experiments, we used P1 cells.

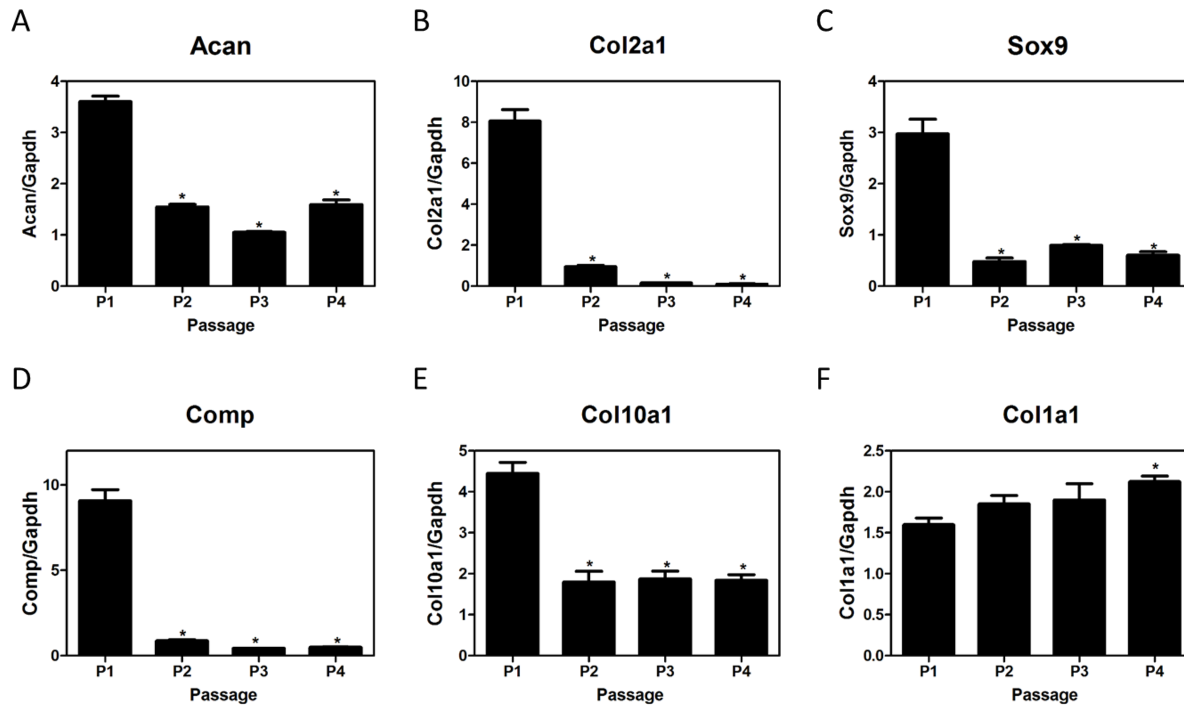

Figure 3.1: Expression of messenger RNA for chondrocyte genes were measured by real-time qPCR of cells cultured to different passages. Expression is normalized to GAPDH. * $p<0.05$ vs. P1.

Treatment with IL-1β had a dose dependent effect on No production; the effect was significant at both 5 ng/ml and 10 ng/ml of IL-1β. NO levels was significance higher with 10 ng/ml IL-1β when compare to 5 ng/ml IL-1β (Figure 3.2A). PGE_2 production and MMP-13 activity increased dose-dependently with IL-1β treatment an effect which was significant at all concentration examined (Figure 3.2B and 3.2C). After incubating cells with 10 ng/ml IL-1β for 24 hours, levels of cell apoptosis marker Caspase-3 was increased (Figure 3.2D). Percentage of fragmented DNA was also increased at this concentration (Figure 3.2E). Treatment with IL-1β at all three doses decreased the mRNA levels of chondrocyte genes: Acan, Comp, Sox9, and Col II (Figure 3.2F, 3.2G, 3.2H, and 3.2I). Treatment with IL-1β had no effect on the levels of Col X (Figure 3.2J). Treatments with IL- 1β for the entire 24 hours or for the last 12 hours of the

experiment both significantly increased the production of nitric oxide (Figure 3.2.1). We decided to use method of treating cells with IL-1β for 24 hours in our future experiments as it increased the most nitric oxide production. Treatment with $24R,25(OH)_2D_3$ for the last 12 hours with I1β.

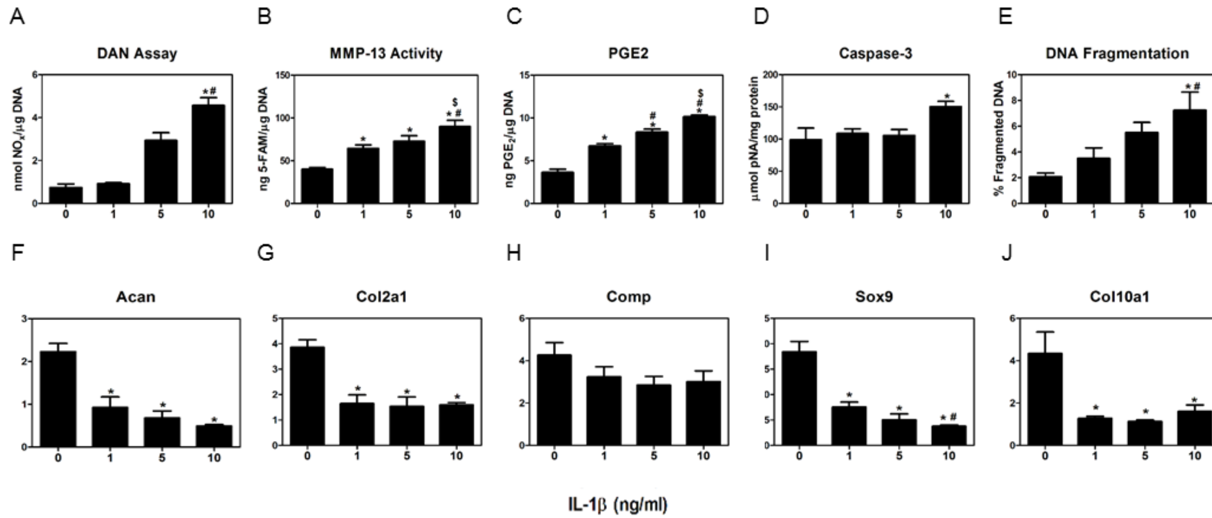

Figure 3.2: Levels of pro-inflammatory and cell apoptosis mediators were measured using ELISA, fluorometric assays, and H3 labeling. Amount is normalized to total DNA. Expression of messenger RNA for P1 chondrocyte genes were measured by real-time qPCR and normalized to GAPDH. * $p<0.05$ vs. control; # $p<0.05$ vs. 1 ng/ml IL-1β; $ $p<0.05$ vs. 5 ng/ml IL-1β.

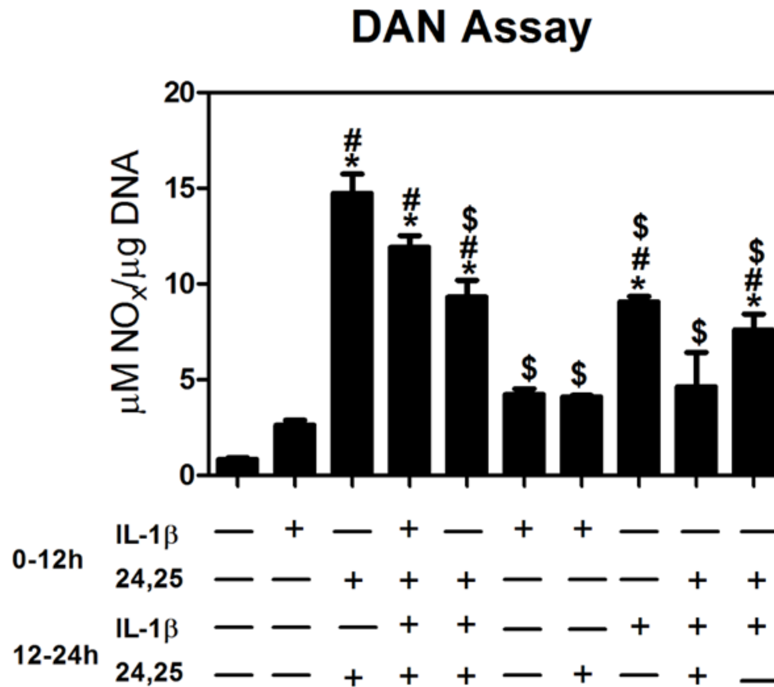

Figure 3.2.1: Levels of nitric oxide measured using ELISA. Amount is normalized to total DNA. Expression of messenger RNA for P1 chondrocyte genes were measured by real-time qPCR and normalized to GAPDH. * $p<0.05$ vs. control; # $p<0.05$ vs. $24R,25(OH)_2D_3$ control; $ $p<0.05$ vs. IL-1β control.

Productions of NO, PGE_2, and MMP-13 was significance enhanced by IL-1β. These effects was dose-dependently inhibited by $24R,25(OH)_2D_3$ treatment it was significance inhibited at $10^{-7}$and 10^{-8} on the NO and MMP-13 production and was significance at all concentration. (Figure 3.3A, 3.3B, and 3.3C). On the mRNA level, treatment with $24R,25(OH)_2D_3$ had no effect on Acan, Col II, and Comp however the inhibition effect of IL-1β was reduced by $24R,25(OH)_2D_3$ in similar way at all concentration examined. (Figure 3.3D, 3.3E, 3.3F).

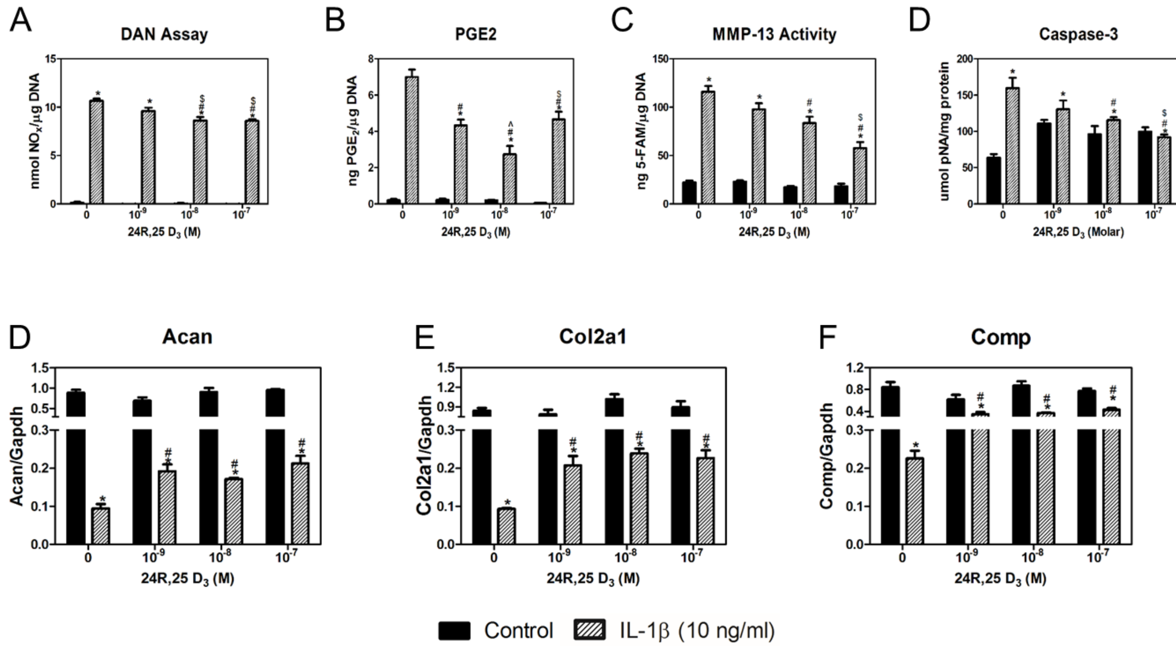

Figure 3.3: A)-C) Nitric oxide, matrix metalloproteinase-13, and prostaglandin E2 levels normalized against DNA. D)-F) Aggrecan, collagen type II, and cartilage oligomatrix protein expression normalized to Gapdh. * $p<0.05$ vs IL-1β control; # $p<0.05$ vs 24R,25(OH)2D3 control; $ $p<0.05$ vs 10^{-8} M; ^ $p<0.05$ vs 10^{-7} M.

Treatment with IL-1β for 24 hours increased the levels of both active and latent TGF-β1with higher stimulation on the active TGF-β1. Treatment with 24R,25$(OH)_2D_3$ increase the amount of active TGF-β1 with no effect on latent TGF-β1. Co-treatment with 24R,25$(OH)_2D_3$ for the last 12 hours further enhanced the production of active TGF-β1 at the 10^{-7}-10^{-8} M concentrations with no effect on latent TGF-β1 (Figure 3.4).

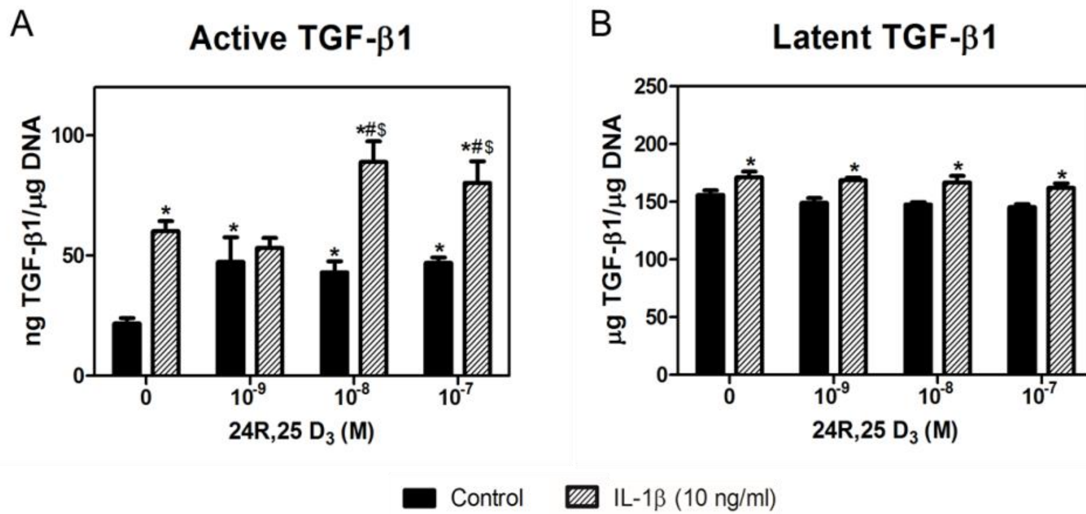

Figure 3.4: Active and latent TGF- β1 levels with IL-1β and $24R,25(OH)_2D_3$ treatment. Levels normalized to total DNA * $p<0.05$ vs. IL-1β control; # $p<0.05$ vs. $24R,25(OH)_2D_3$ control; $ $p<0.05$ vs. 10^{-9} M.

IL-1β decreased Smad2 mRNA levels(5 ng/ml) and increased the mRNA levels of Smad 3 and decreased the levels of Tgfbr2 at the 10 ng/ml concentration after 12 hours of treatment (3.5A, 3.5B, 3.5C). The inhibition of the Addition of $24R,25(OH)_2D_3$ for the last 12 hours of treatment with IL-1β increased mRNA levels of Smad2 and Smad3 at the 10^{-8} M concentration and increased the levels of Tgfbr2 at both 10^{-8} M and 10^{-9} M concentrations (Figure 3.5D, 3.5E, 3.5F).

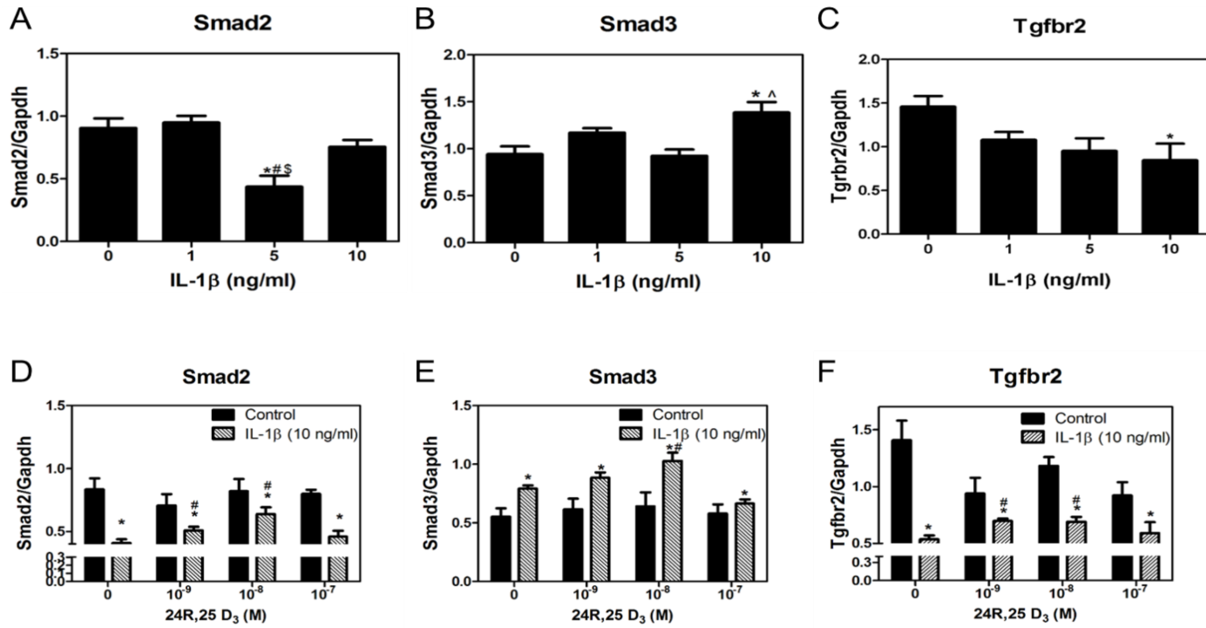

Figure 3.5: A)-C) Effect of IL-1β on TGF- β1 signaling molecules Smad 2/3 and receptor Tgfbr2. * p<0.05 vs. control; # p<0.05 vs. 1 ng/ml; ^ p<0.05 vs. 5 ng/ml; $ p<0.05 vs. 10 ng/ml. D-F) Effect of 24R,25$(OH)_2D_3$ on TGF- β1 signaling molecules Smad 2/3 and receptor Tgfbr2. * p<0.05 vs. control; # p<0.05 vs. IL-1β control.

Treatment with TGF-β1 at 0.1, 1, and 10 ng/ml concentrations for 24 hours showed a dose dependently decreased NO and MMP-13 production which was enhanched by IL-1β (3.6A and 3.6B). On PGE_2 production the inhibition effect of IL-1β was found only at 0.1 ng/ml (Figure 3.6C).

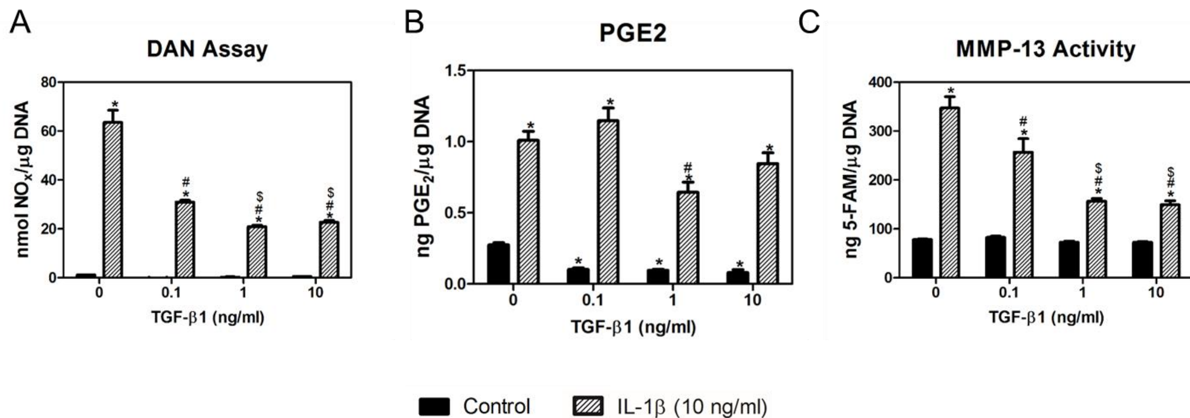

Figure 3.6: Effect of TGF- β1 treatment on nitric oxide, prostaglandin E2, and matrix metalloproteinase-13 levels. Protein levels normalized against total DNA * $p<0.05$ vs. control; # $p<0.05$ vs IL-1β control; $ $p<0.05$ vs. 0.1 ng/ml.

Co-treatments with 10^{-8} M 24R,25$(OH)_2D_3$ and 0.1 ng/ml TGF-β1 additively decreased the production of MMP-13 compare to the effect of TGF-β1 or 24R,25$(OH)_2D_3$ (Figure 3.7A). Treatment with antibody against TgfbrII did not have an effect on MMP-13 production by IL-1β, moreover the inhibition effect of 24R,25$(OH)_2D_3$ on the IL-1β on MM-13 was not affected by the antibody against TgfbrII (Figure 3.7B) . Similarly treatment with the soluble receptor TgfbrII had no effect on MMP-13 production with or without 24R,25$(OH)_2D_3$ (Figure 3.7C).

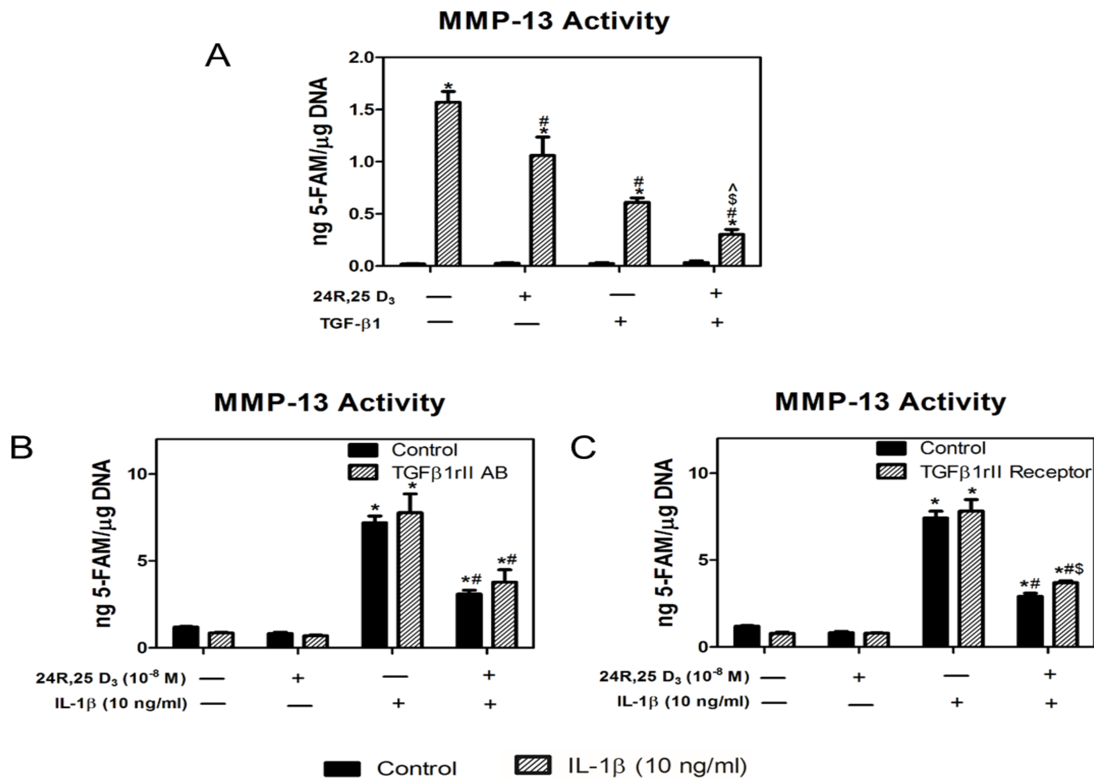

Figure 3.7: A) Effect of 0.1 ng/ml TGF- β1 with $24R,25(OH)_2D_3$ co-treatment on IL-1β induced MMP-13 production. Levels normalized to total DNA * $p<0.05$ vs. IL-1β control; # $p<0.05$ vs. $24R,25(OH)_2D_3$ control; \$ $p<0.05$ vs. 10^{-8} M $24R,25(OH)_2D_3$; ^ $p<0.05$ vs. 0.1 ng/ml TGF-β1. B-C) Effect of blocking TGF-β1 signaling on $24R,25(OH)_2D_3$ reducing MMP-13 production. Levels normalized to total DNA * $p<0.05$ vs. IL-1β control; # $p<0.05$ vs. $24R,25(OH)_2D_3$ control; \$ $p<0.05$ vs. 10^{-8} M

Treatment of the human osteoarthritic chondrocytes with $24R,25(OH)_2D_3$ lowered alkaline phosphatase activity in the erosion cells compared to the control and compared to the minimal-fibrillated cells (Figure 8A). $24R,25(OH)_2D_3$ increased the mRNA levels of ACAN and COL II in the minimal-fibrillated cells compared to the control, and these levels were significantly higher compared to the erosion cells (Figure 8B, 8C, 8D).

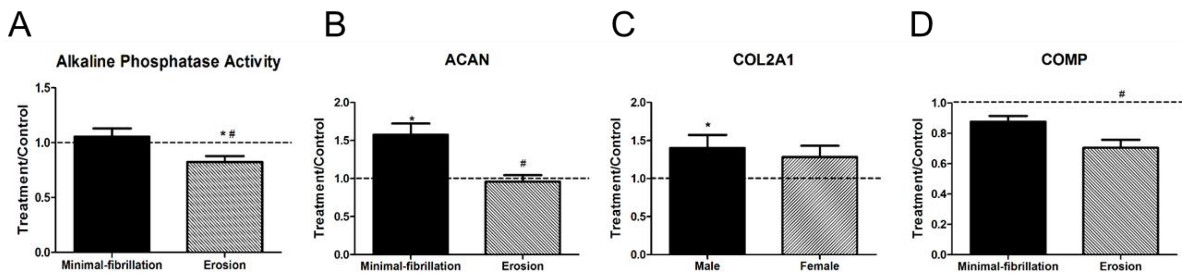

Figure 3.8: Response of passage 1 chondrocytes treated with 10^{-7} M $24R,25(OH)_2D_3$. Alkaline phosphatase activity was measured in whole cell lysates (A). mRNAs of chondrocyte genes were measured using RealTime PCR (B-D). Data show treatment vs. vehicle control ratios. The dashed line represents the vehicle control (dashed line=1). *$P<0.05$ vs. Control; # vs. Male.

DISCUSSION

We established a system that mimics OA-like symptoms by stimulating p1 rat articular chondrocytes with different concentrations of rat IL-1β. We assessed the establishment of the OA model based on inflammatory factors, chondrocyte apoptosis, and matrix degradation. Our results show that not only IL-1β increased the production of NO and PGE2, which are inflammatory factors involved in OA. IL-1β treatment increased cell apoptosis as it increased

DNA fragmentation and Caspase-3 activity. The observation of increased levels of nitric oxide coupled with an increase in caspase-3 activity and DNA fragmentation suggest that there are elevated apoptosis processes. In addition, IL-1β decreased the mRNA levels of chondrocyte genes that constitute cartilage matrix and increased matrix metalloproteinase MMP-13 activity, which further compromises cartilage matrix integrity MMPs are families of proteolytic enzymes, which are normally required for timely and controlled degradation of extracellular matrix components during tissue remodeling [68, 69, 71, 87, 162]. Results from other studies corroborate our findings as IL-1β has been shown to induce inflammation and cartilage degradation therefore mimics the OA microenvironment *in vitro*.

In this study, we evaluated the therapeutic potential of $24R,25(OH)_2D_3$ in an OA cell culture model stimulated by IL-1β. Our preliminary results show that when added to the media in the last twelve hours of IL-1β treatment, 10^{-8} M of $24R,25(OH)_2D_3$ significantly decreased the production of NO by 40%. Therefore, we chose to use this treatment method for our following studies. In our study, $24R,25(OH)_2D_3$ treatment decreased NO and PGE2 levels, and MMP-13 activity, induced by IL-1β. Previously, $24R,25(OH)_2D_3$ has also been shown to regulate MMP-3 production in rat resting zone chondrocytes [124]. NO and PGE_2 are known to be important inflammatory mediators in OA pathogenesis. NO mediates other pro-inflammatory cytokines. The effect of $24R,25(OH)_2D_3$ on reducing IL-1β induced NO and PGE_2 production is promising for alleviating the inflammatory environment in OA. These results collectively suggest that $24R,25(OH)_2D_3$ may promote cartilage regeneration by reducing the levels of catabolic factors that matrix enzyme activity. $24R,25(OH)_2D_3$ also increased the mRNA levels of chondrocyte genes that were down-regulated by IL-1β. Since alterations in ECM genes can play an important role in maintaining osteoarthritic pathogenesis, our results suggest an $24R,25(OH)_2D_3$ treatment

prevents the loss of chondrocyte phenotype, and further enhances cartilage matrix repair to prevent the progression of OA. In human osteoarthritic chondrocytes, $24R,25(OH)_2D_3$ treatment up-regulated the mRNA levels of ACAN and COL2A1 and had no effect on alkaline phosphatase activity in minimal fibrillated chondrocytes, suggesting that $24R,25(OH)_2D_3$ can enhance chondrocyte phenotype without stimulating differentiation. In contrast, treatment of $24R,25(OH)_2D_3$ on cells obtained from erosion site decreased alkaline phosphatase activity while having no effect on chondrocyte phenotype, illustrating that $24R,25(OH)_2D_3$ may have lost its protective effect on the eroded chondrocytes. Results from our study demonstrate that $24R,25(OH)_2D_3$ treatment exhibited protective effects against osteoarthritic changes in an in vitro model. This is supported by data that showed OA chondrocytes lose their ability to respond to $24R,25(OH)_2D_3$ when they are in an advanced disease state. Results from this study suggest that $24R,25(OH)_2D_3$ could potentially be used as a therapeutic treatment for knee osteoarthritis.

To assess the effect of $24R,25(OH)_2D_3$ treatment, we compared our results to a growth factor that has been shown to promote chondrocyte growth and differentiation. TGF-β1 can stimulate the production of cartilage ECM by chondrocytes and could counteract IL-1β-induced tissue degradation [95]. Our results show that TGF-β1 decreased the production of NO, PGE_2, and MMP-13; similar to the effects of $24R,25(OH)_2D_3$. Since TGF-β1 is known as an potential inhibitor of OA, these results suggest that $24R,25(OH)_2D_3$ could be used to halt OA disease progression without the side effects of using a growth factor that can cause liver damage.

To elucidate the pathway of $24R,25(OH)_2D_3$'s action, we treated chondrocytes with IL-1β with various concentrations of $24R,25(OH)_2D_{3.}$ Ours results show that TGF-β1 levels were up-regulated by both IL-1β and $24R,25(OH)_2D_{3.}$ The increase in active TGF-β1 levels by IL-1β is consistent with literature findings that TGF-β1 expression is increased in the early hypertrophic

phase of the altered cartilage [14]. 24R,25$(OH)_2D_3$ treatment increased the levels of active TGF-β1, which is the form that exerts the chondrocyte protective effect, suggesting that 24R,25$(OH)_2D_3$ regulates TGF-β1. To investigate further how the TGF-β1 pathway is regulated, we measured the expression of TGF-β1 signaling molecules after IL-1β and 24R,25$(OH)_2D_3$ treatments. Smads 2 and 3 are signaling transduction molecules for the TGF-β1 pathway [100]. In our study, IL-1β down-regulated the mRNA levels of Smad 2 and up-regulated that of Smad3. These results are consistent with literature; positive and negative effects of IL-1β on Smad signaling have been reported, suggesting that OA pathogenesis is a complex process. Studies have shown that IL-1 induced a local Smad3-dependent tissue fibrosis when over-expressed in the murine lung [11]. Modulation of TGF-β1 signaling by IL-1β may play a significant role in determining chondrocyte cell response to injury and cartilage repair in OA. However, IL-1β down-regulated the mRNA levels of TGF-β1 receptor, suggesting that OA chondrocytes are becoming insensitive to the growth factor and may not be able to prevent cartilage degradation induced by IL-1β. 24R,25$(OH)_2D_3$ increased the mRNA levels of Smad2, Smad3 and Tgfbr2, suggesting that there are potential interactions between the two molecules.

Co-treatments with 24R,25$(OH)_2D_3$ and TGF-β1 synergistically decreased the production of MMP-13. Since the two molecules showed similar protective effects in our OA model, we investigated if TGF-β1 is mediating the effect of 24R,25$(OH)_2D_3$ in blocking OA likes changes in our system. Blocking TGF-β1 signaling with AB against TGF-β1 and with a decoy receptor for Tgfβ1rII did not have an effect of MMP-13 production. These data suggest that the effect exhibited by 24R,25$(OH)_2D_3$ on counteracting IL-1β induced MMP-13 production is not mediated through the TGF-β1 signaling pathway.

Collectively, these data suggest that 24R,25$(OH)_2D_3$ plays an important role in protection

of chondrocyte from undergoing hypertrophic changes, which can lead to cell apoptosis, articular cartilage degeneration, and progression of OA. $24R,25(OH)_2D_3$ has shown to modulate and enhance the effect of TGF-β1, suggesting that $24R,25(OH)_2D_3$ may be used as a promising inhibitor of IL-1β induced production of cartilage catabolic and inflammatory factors known to be involved in OA pathogenesis.

CONCLUSION

Results from our study demonstrate that $24R,25(OH)_2D_3$ treatment exhibited protective effects on osteoarthritic chondrocytes. Mechanism of $24R,25(OH)_2D_3$'s action is through inhibiting production of IL-1β induced catabolic factors. In addition, although there exhibits an addictive effect between $24R,25(OH)_2D_3$ and TGF-β1, the effect of $24R,25(OH)_2D_3$ is not mediated through the TGF- β pathway. Furthermore, OA chondrocytes lost their ability to respond to $24R,25(OH)_2D_3$ when they are in an advanced disease state.

CHAPTER 4

24R,25(OH)2D3 AMEOLIORATES ARTICULAR CARTILAGE DEGRADATON IN A RAT ACLT MODEL OF OSTEOARTHRITIS

INTRODUCTION

Osteoarthritis is a degenerative disease that affects all components of the joint. Translational research is a critical step towards understanding and mitigating the long-term effects of this disease process. Surgical models of mechanical instability appear to represent chronic traumatic OA. In this study, anterior cruciate ligament transection (ACLT) was performed to induce OA in the rat to study the histological and biochemical changes occurring during OA progression. 24R,25$(OH)_2D_3$ has been shown to play important roles in cartilage growth and remodeling. The aim of this study is to investigate the therapeutic effects of 24R,25$(OH)_2D_3$ in mediating ACLT-induced joint pathologies in rat as assessed by Toluidine blue staining of joint sections and disease severity scoring using a semi-quantitative grading system.

MATERIALS AND METHODS

ACLT model of OA

20 male Sprague-Dawley rats weighing 275-300 grams were divided into two groups. In each group, the left leg of the knee served as a contralateral control. Animals undergone surgery for ACLT under isoflurane anesthesia. On day 0, a parapatellar skin incision is performed on the medial side of the right knee joint and then on the medial side of the patellar tendon. The patella is then dislocated laterally to provide access to the joint space and the anterior cruciate ligament

is transected in the flexed knee. A positive anterior drawer test is performed to confirm complete transection of the ligament. The joint is then irrigated with sterile saline to avoid ancillary inflammation, and a purpose made suture is inserted. Buprenorphine was administered as analgesic on the day of surgery (during surgery and a second dose after 8-12h).

Intraarticular Injection of 24R,25(OH)$_2$D$_3$

24R,25(OH)$_2$D$_3$ was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA) and dissolved in ethanol with a stock concentration of 10^{-4} M. 40 ul of the diluted 24R,25(OH)$_2$D$_3$ was then dissolved in 10 ml of sterile 1x phosphate buffered saline, resulting in a final concentration of $4*10^{-7}$ M 24R,25(OH)$_2$D$_3$. 24R,25(OH)$_2$D$_3$ or vehicle (ethanol dissolved in sterile 1x phosphate buffered saline) was injected immediately after the ACLT surgery by irrigating the articular space with test item formulation using a micro-pipette. 25 ul of solution was injected into each animal per dosing. Subsequent injections were done every 7 days until day 28. During injection, animal are maintained under general anesthesia with isoflurane. After anesthesia is achieved, the animal is restrained in a dorsal recumbent position; the hind limb articulation is shaved and wiped generously with alcohol solution to facilitate the localization of the injection/collection site. The injection site was examined grossly by palpation of the tibia head and the patellar ligament, to identify the injection site. The needle was inserted above the tibia head and behind the patellar ligament. The injection was performed with a 25G needle by slowly releasing of the test item into the articular space.

Blood serum and synovial fluid collection

Blood was collected terminally on Day 28 as well as via jugular puncture on Day 1, 8, 15 and 22 post-ACLT. Terminal bleeds was done under isoflurane anesthesia and blood (~1 mL)

and was collected via abdominal aorta following which animals was euthanized by exsanguination of the abdominal aorta. All blood samples were processed for serum using standard serum separator tubes (without EDTA). On Day 28, synovial fluid lavage from both knee joints was collected following the terminal blood collection and immediately before the knee joint collection. Injection of lavage fluid (200 µl) was done following the intra-articular injection procedure as described in section 2.2. The limb was flexed and extended several times and lavage fluid was drawn by re-inserting the needle at the same location.

Histology

Immediately after the synovial fluid collection, intact right and left knee joints were harvested and fixed in neutral buffered 10% formalin separately for histopathological analysis. Whole knee joints were decalcified (Decal Chemical Corporation, Tallman, NY, USA) for 16 hours on a rotating platform before being dehydrated in a series of 95% and 100% ethanol and xylene washes. Samples were embedded in paraffin and cut into 7-µm sections (Shandon Fillesse 325, Thermo Scientific). Toluidine blue and Safranin-O staining were completed for each sample. Samples were imaged using a Zeiss Observer Z1 using a 20x objective. Modified Mankin score system was used for semi-quantitative histopathology grading (Table 4.1). Individual score was recorded for each sample in an Excel spreadsheet.

ELISA

Levels of inflammatory factors involved in OA and TGF-βs (1, 2, and 3) in the synovial fluid and serum were measured using a magnetic bead–based multiplex assays (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Data are presented as mean ± SEM of n=10 animals per group. Data were first examined by analysis of variance (ANOVA) and post hoc test using Bonferroni's modification of Student's t-test. $P<0.05$ was considered to be significant.

RESULTS

Table 4.1 Modified Mankin Score

I. Structural changes (0-10)	
Normal	0
Surface irregularities (Undulating articular surface but no fibrillation)	1
Minimal mild superficial fibrillation (less than 10% of articular cartilage thickness) < 50% of the plateau/condyle surface	2
Minimal mild superficial fibrillation(less than 10% of articular cartilage thickness) > 50% of the plateau/condyle surface	3
Fibrillation/clefts/fissure/loss of articular cartilage involving superficial 1/3 of articular cartilage < 50% of the plateau/condyle surface	4
Fibrillation/clefts/fissure/loss of articular cartilage involving superficial 1/3 of articular cartilage > 50% of the plateau/condyle surface	5
Fibrillation/clefts/fissure/loss of articular cartilage involving superficial 1/3 to 2/3 of articular cartilage < 50% of the plateau/condyle surface	6
Fibrillation/clefts/fissure/loss of articular cartilage involving superficial 1/3 to 2/3 of articular cartilage > 50% of the plateau/condyle surface	7
Fibrillation/clefts/fissure/loss of articular cartilage involving superficial > 2/3 of articular cartilage < 50% of the plateau/condyle surface	8
Fibrillation/clefts/fissure/loss of articular cartilage involving superficial > 2/3 of articular cartilage >50% of the plateau/condyle surface	9
Fibrillation/clefts/fissure/loss of articular cartilage to subchondral bone	10
II. Toluidine blue staining (0-6)	
Normal	0
Loss of staining in superficial zone of articular cartilage involving < 50% plateau/condyle	1
Loss of staining in superficial zone of articular cartilage involving ≥ 50% plateau/condyle	2
Loss of staining in upper 2/3 of articular cartilage involving < 50% plateau/condyle	3
Loss of staining in upper 2/3 of articular cartilage involving ≥ 50% plateau/condyle	4
Loss of staining in all the articular cartilage involving < 50% plateau/condyle	5
Loss of staining in all the articular cartilage involving > 50% plateau/condyle	6
III. Clones (cluster) formation (0-3)	
None	0
< 4 clones	1
≥4 but <8 clones	2
≥8 clones	3
IV. Loss of chondrocytes (0-6)	
Normal	0
Focal chondrocyte loss	1
Loss of chondrocytes in superficial zone <50% of the condyle/plateau	2
Loss of chondrocytes in superficial zone >50% of the condyle/plateau	3
Loss of chondrocytes in mid zone <50% of the condyle/plateau	4
Loss of chondrocytes in mid zone >50% of the condyle/plateau	5
Diffuse loss of chondrocytes	6

Histology

Both contralateral control knees showed intact toluidine blue staining (Figure 4.1A, 4.1B). In the ACLT group, vehicle treated knees showed significant loss in toluidine blue staining indicating loss of proteoglycan and cartilage tissue (Figure 4.1C). Toluidine blue staining was greater in 24R,25$(OH)_2D_3$ treated knees compared to vehicle-treated knees and was comparable to un-operated articular cartilage (Figure 4.1D). Histopathological scoring using a modified-Mankin scoring system was used to grade joint show that 24R,25$(OH)_2D_3$ treatment signifincanly reduced toluidine score in the medial femur, lateral femur and medial tibia compared to the vehicle treated control (Figure 4.1E, 4.1F, 4.1G). There was no significant difference between 24R,25$(OH)_2D_3$ and vehicle treatment in lateral tibia (Figure 4.1H).

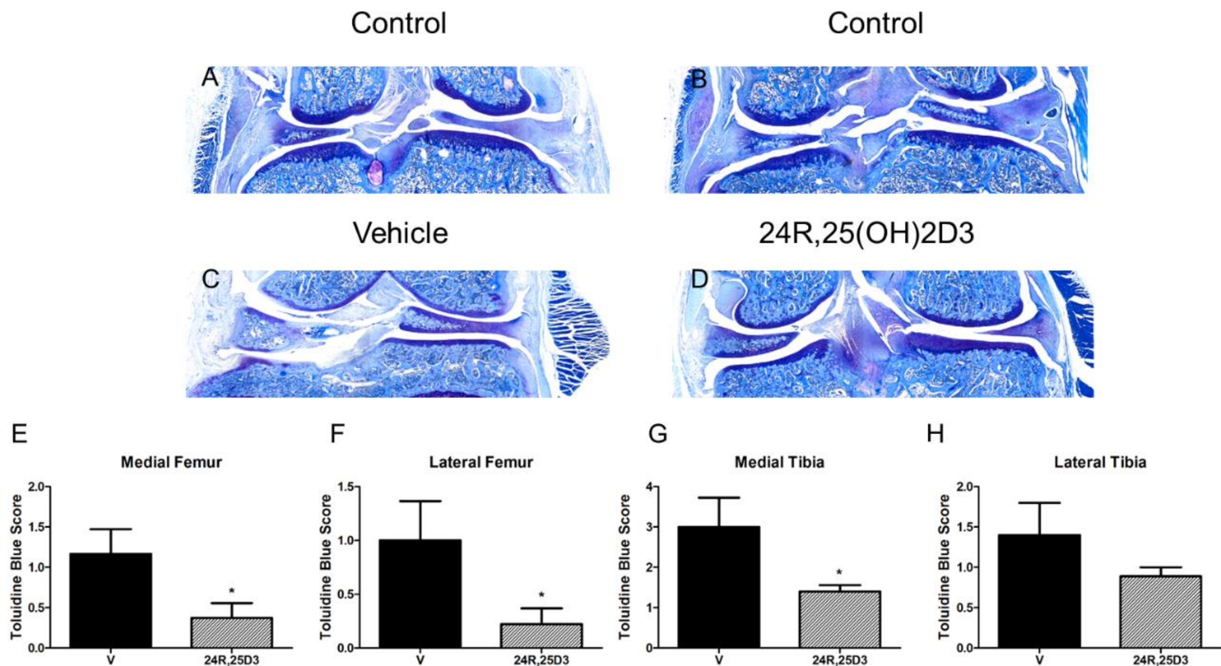

Figure 4.1: Toluidine blue staining of intact articular joint (A-D) and pathology scores for the four quadrants of the knee joint. * $p < 0.05$ vs. vehicle treated.

Safranin-O staining showed both significant proteoglycan loss in vehicle knees compared to both un-operated contralateral knees (Figure 4.2A, 4.2B, 4.2C). 24R,25$(OH)_2D_3$ treatment reduced proteoglycan compared to the vehicle treated knees (Figure 4.2D). Histopathological scoring using a modified-Mankin scoring system was used to grade joint show that 24R,25$(OH)_2D_3$ treatment significantly reduced toluidine score in the medial femur, lateral femur and medial tibia compared to the vehicle treated control (Figure 4.2E, 4.2F, 4.2G). There was no significant difference between 24R,25$(OH)_2D_3$ and vehicle treatment in lateral tibia (Figure 4.2H).

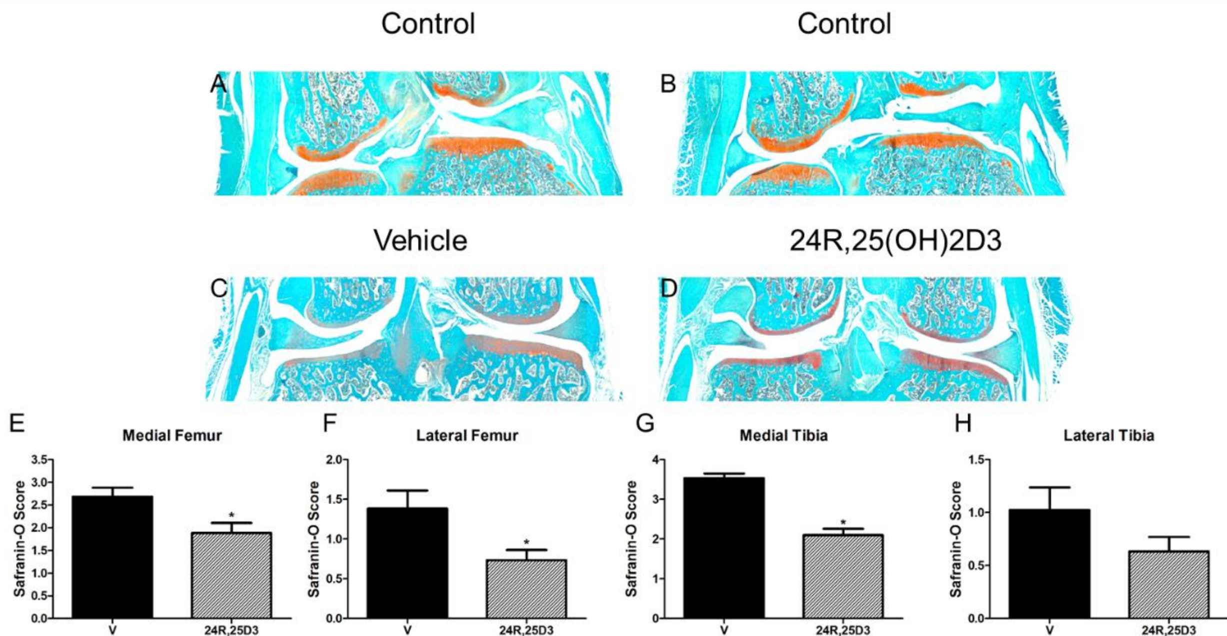

Figure 4.2: Safranin-O staining of intact articular joint (A-D) and pathology scores for the four quadrants of the knee joint. * $p < 0.05$ vs. vehicle treated.

Total composite scores consists of structural changes, toluidine blue staining, Clones (cluster) formation, and chondrocyte cell loss (in our study, the changes are mainly due to

structural changes and toluidine blue changes). Total composite score shows a moderate OA induction in the ACLT operated groups. $24R,25(OH)_2D_3$ significantly lowered total composite scores for medial femurs, medial tibias and lateral femurs compared to vehicle, indicating less OA damage (Figure 4.3A, 4.3B. 4.3C). $24R,25(OH)_2D_3$ decreased cell clustering, chondrocyte loss, and cartilage fibrillation. There was no significant difference between $24R,25(OH)_2D_3$ and vehicle treated in lateral tibia (Figure 4.3D).

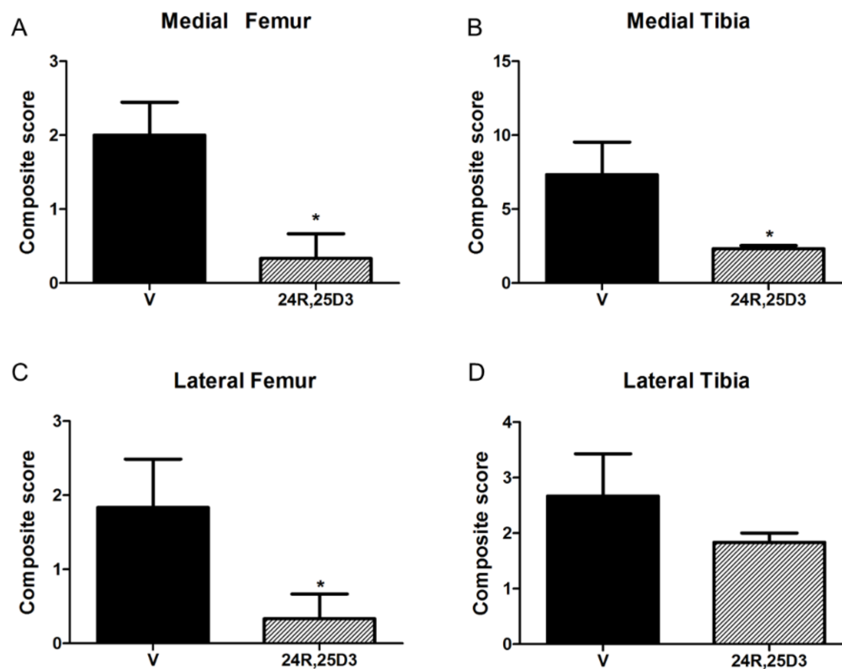

Figure 4.3: Total scores for the four quadrants of the knee joint. * $p < 0.05$ vs. vehicle treated.

Synovial Fluid

No statistical differences were observed in levels of granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) between vehicle treated and 24R,25$(OH)_2D_3$ treated group (Figure 4.4A, 4.4B). In the ACLT operated group, vehicle treated group had increased levels of G-CSF and GM-CSF compared to the no-operated groups. 24R,25$(OH)_2D_3$ decreased levels of G-CSF and GM-CSF (Figure 4.4A, 4.4B) compared to the vehicle treated control and restored the levels of GM-CSF to un-operated control levels.

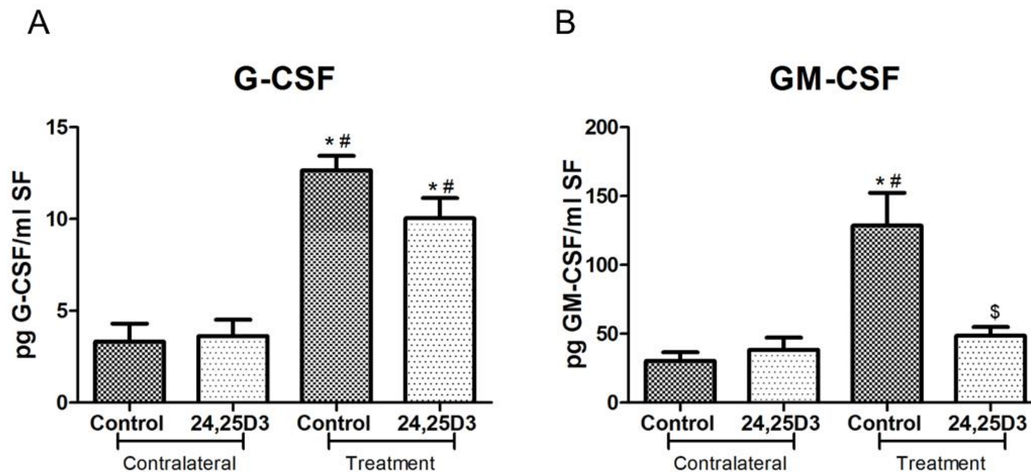

Figure 4.4: Synovial fluid levels of macrophage colony stimulating factors. * $p < 0.05$ vs. contralateral control; # $p < 0.05$ vs. 24,25D_3 contralateral control; $ $p < 0.05$ vs. vehicle treatment.

Levels of growth related oncogene (GRO), macrophage inflammatory protein 3α (MIP3α) were increased in the ACLT operated groups compared to both contralateral controls. Treatment of 24R,25$(OH)_2D_3$ decreased levels of GRO and MIP3α to contralateral control level (Figure 4.5A, 4.5C). Macrophage inflammatory protein 1α (MIP1α) and chemokine (C-C motif)

ligand 5 (RANTES) were not detected in the synovial fluid (Figure 4.5B, 4.5E). No differences in levels of monocyte chemoattractant protein-1 (MCP-1) were observed between control and ACLT operated groups (Figure 4.5D).

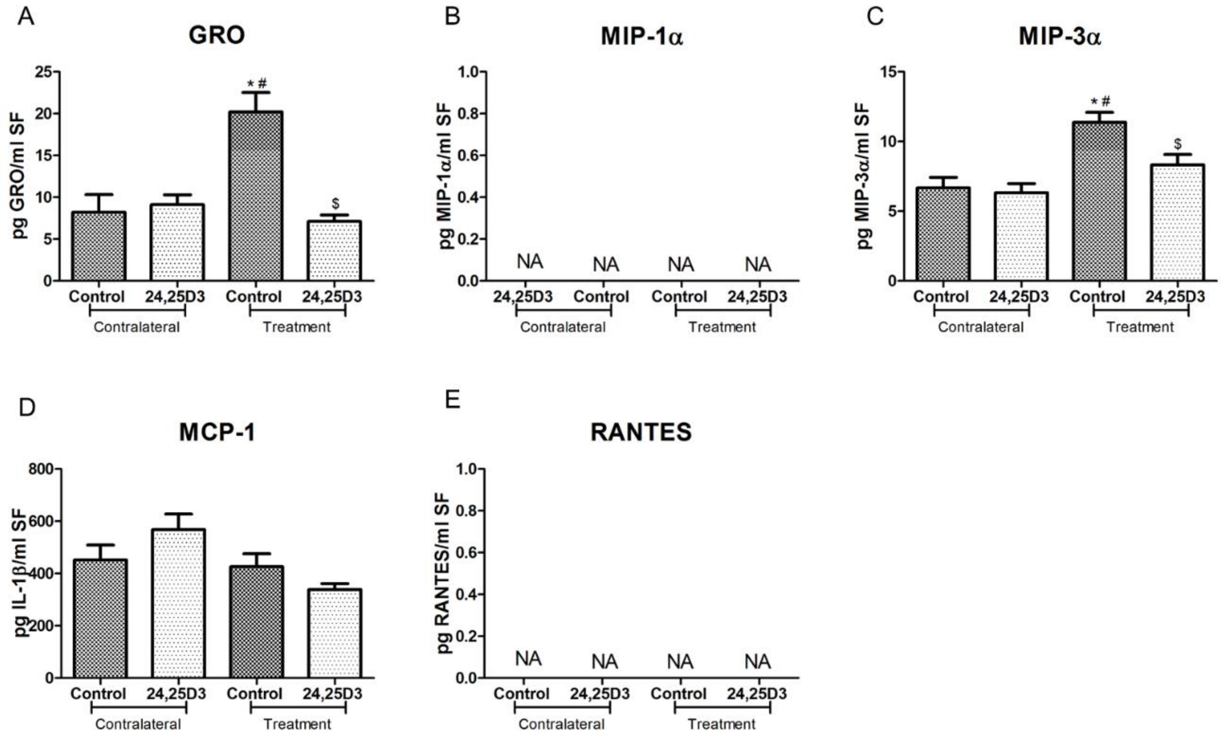

Figure 4.5: Synovial fluid levels of chemokines. * $p < 0.05$ vs. contralateral control; # $p < 0.05$ vs. 24,25D$_3$ contralateral control; $ $p < 0.05$ vs. vehicle treatment.

Pro-inflammatory cytokine levels were measured. In the ACLT operated group, levels of interleukin 1α, 1β, 2, 6, 7 (IL-1α, IL-1β, IL-2, IL-6, IL-7) were increased compared to both contralateral controls. 24R,25$(OH)_2D_3$ treatment decreased levels of IL-1α, IL-1β and IL-7 compared to the vehicle treatment (Figure 4.6A, 4.6B, 4.6E) and brought levels of IL-1β to control level. There were no significant differences in level of IL-2 between the control group and ACLT group (Figure 4.6C). 24R,25$(OH)_2D_3$ treatment did not have an effect on IL-6 levels (Figure 4.6D).

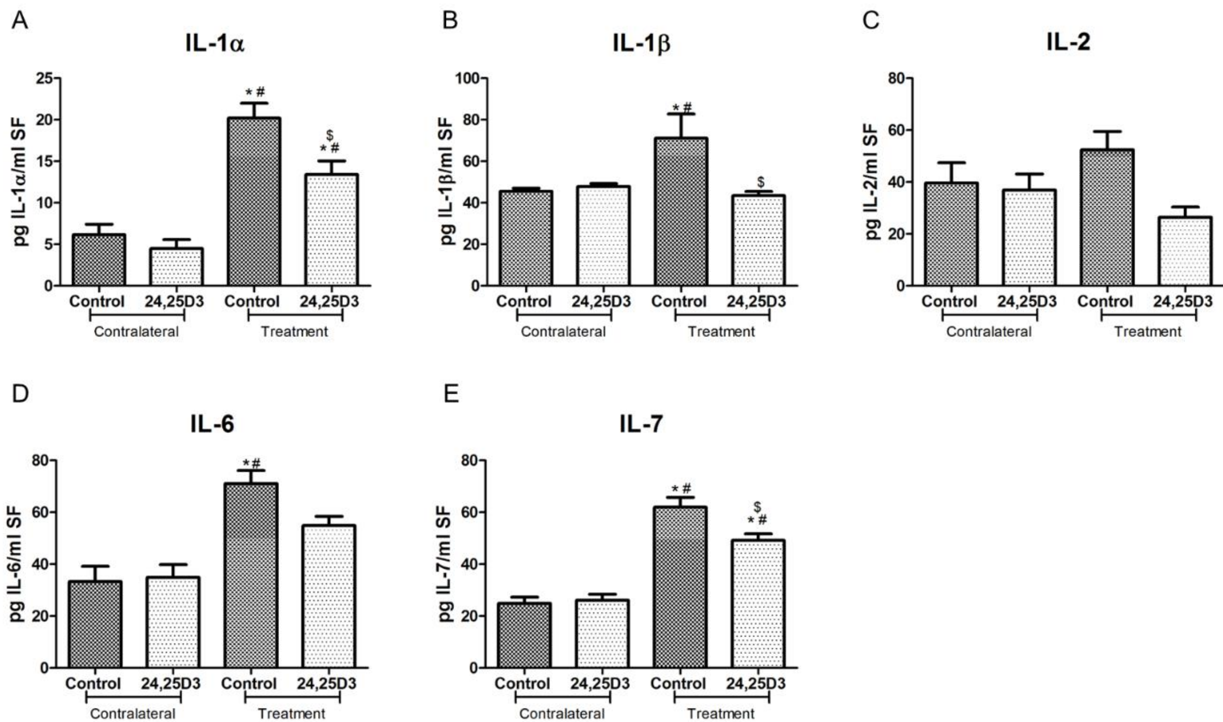

Figure 4.6: Synovial fluid levels of pro-inflammatory cytokines. * $p < 0.05$ vs. contralateral control; # $p < 0.05$ vs. 24,25D_3 contralateral control; $ $p < 0.05$ vs. vehicle treatment.

Inflammatory mediators interleukin 12, 17 and 18 (IL-12, IL-17 and IL-18), as well as interferon-gamma (IFN-g) and tumor necrosis factor alpha (TNF-α) levels were increased in vehicle treated ACLT knees compared to both contralateral controls (Figure 4.7A, 4.7B, 4.7C,

4.7D, 4.7E). 24R,25$(OH)_2D_3$ treatment did not have an effect on IL-12 and it significantly decreased levels of IL-17, IL-18, IFN-g and TNF-α (Figure 4.7A, 4.7B, 4.7C, 4.7D, 4.7E). In addition, treatment of 24R,25$(OH)_2D_3$ was able to bring the levels of IL-18 and TNF-α back to control levels.

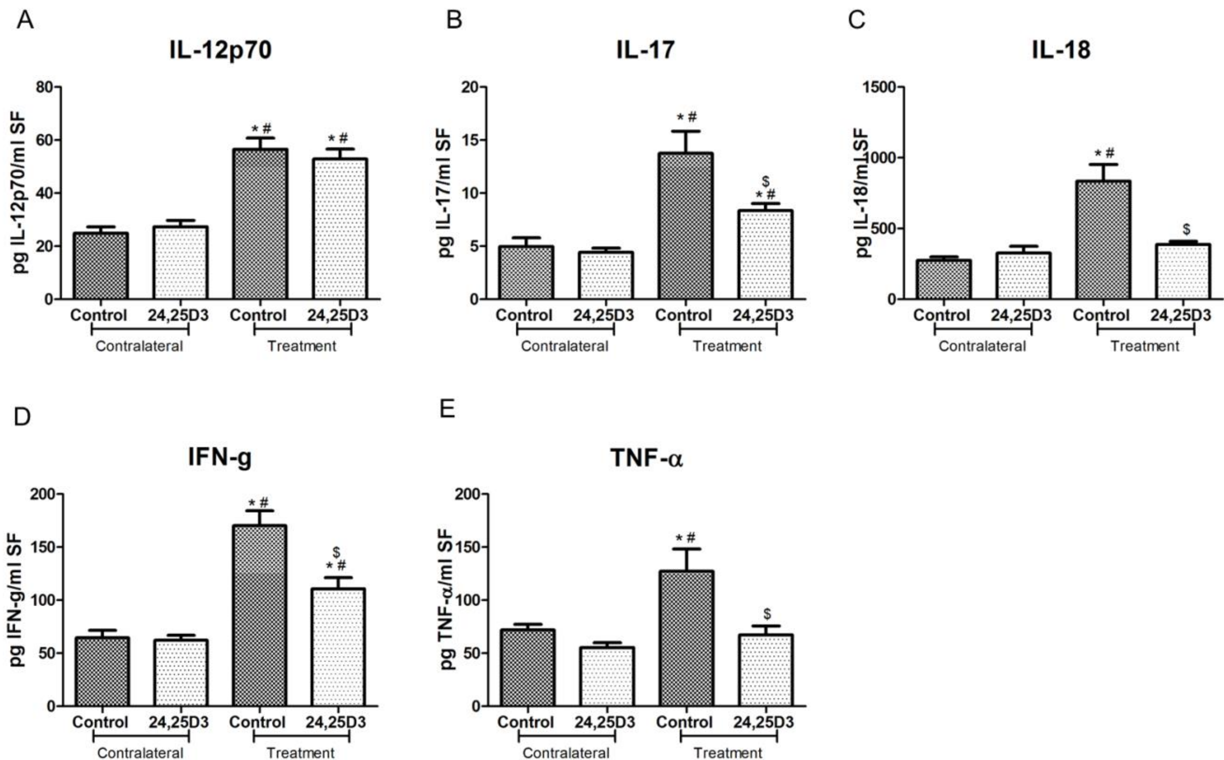

Figure 4.7: Synovial fluid levels of inflammatory factors. * $p < 0.05$ vs. contralateral control; # $p < 0.05$ vs. 24,25D_3 contralateral control; $ $p < 0.05$ vs. vehicle treatment.

In the ACLT operated group, vehicle treated knees had lower levels of anti-inflammatory factors. Specifically, levels of interleukin 4, 5, 10, 13 and macrophage colony-stimulating factor (IL-4, IL-5, IL-10, IL-13 and M-CSF) were significantly lower compared to levels of the control knees (Figure 4.8A, 4.8B, 4.8C, 4.8D, 4.8E). Treatment of 24R,25$(OH)_2D_3$ had no effect on IL-4 levels (Figure 4.8A) and it increased levels of IL-5, IL-10, IL-13 and M-CSF (4.8B, 4.8C, 4.8D, 4.8E).

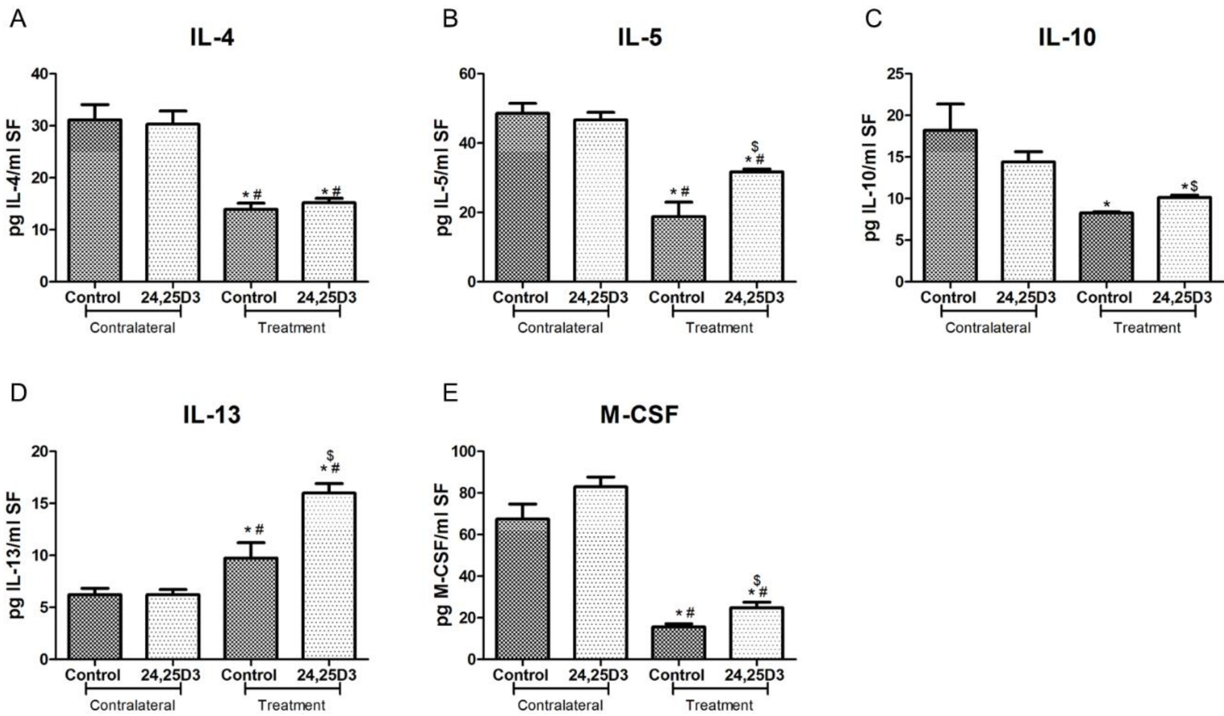

Figure 4.8: Synovial fluid levels of anti-inflammatory factors. * $p < 0.05$ vs. contralateral control; # $p < 0.05$ vs. $24,25D_3$ contralateral control; $ $p < 0.05$ vs. vehicle treatment.

Transforming growth factor β1 (TGF-β1) levels were lower in vehicle treated ACLT knees compared to controls. Levels of TGF-β1 were higher in $24R,25(OH)_2D_3$ treated groups (Figure 4.9A). Vehicle treated knees had higher levels of TGF-β2 and 3 compared to controls and there were no significant differences between vehicle and $24R,25(OH)_2D_3$ treatment (Figure 4.9B and 4.9C). Vascular endothelial growth factor (VEGF) levels were higher in both treatment groups compared to controls and $24R,25(OH)_2D_3$ treatment reduced this increase compared to vehicle treated (Figure 4.9D). Erythropoietin (EPO) levels were lower in vehicle treated group compared to the control and compared to $24R,25(OH)_2D_3$ treated group. $24R,25(OH)_2D_3$ treatment up-regulated levels of EPO to control level (Figure 4.9E).

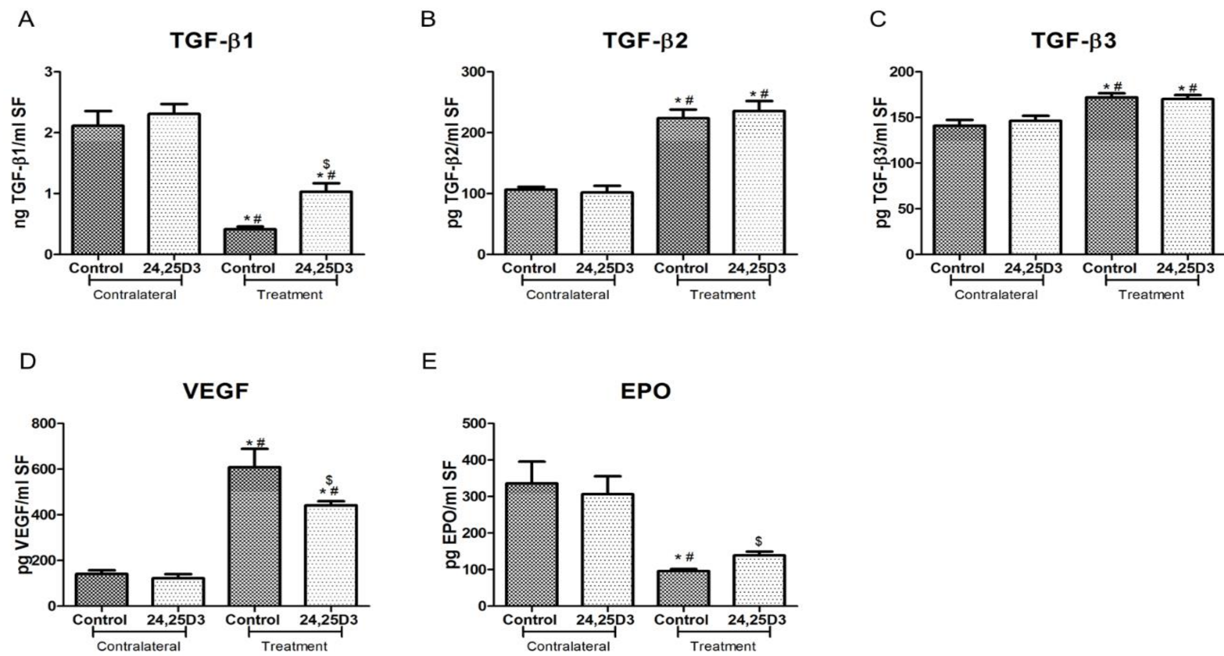

Figure 4.9: Synovial fluid levels of growth factors protein. * $p < 0.05$ vs. contralateral control; # $p < 0.05$ vs. $24,25D_3$ contralateral control; $ $p < 0.05$ vs. vehicle treatment.

Serum

Levels of G-CSF and GM-CSF were higher at day 28 than day 1 levels. Treatment of 24R,25$(OH)_2D_3$ lowered the levels of G-CSF and GM-CSF compared to the vehicle treated at day 28 (Figure 4.10A, 4.10B).

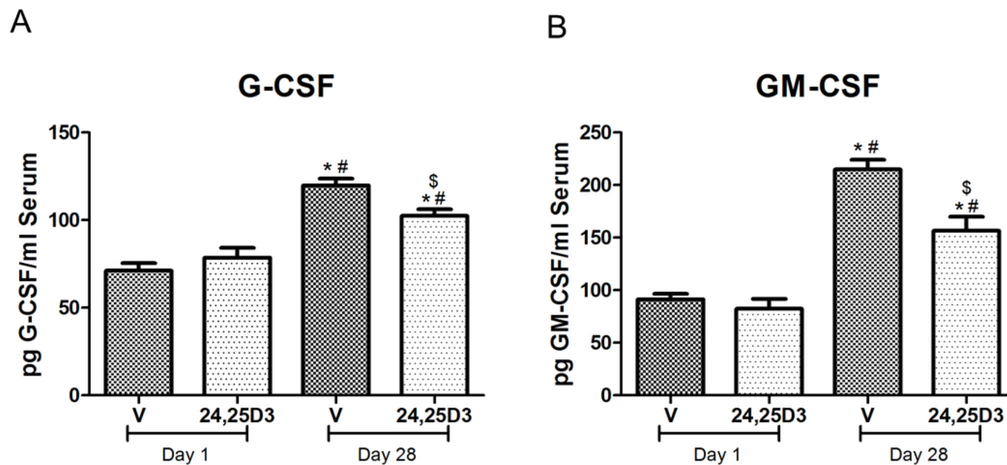

Figure 4.10: Serum levels of macrophage colony stimulating factors. * $p < 0.05$ vs. contralateral control; # $p < 0.05$ vs. 24,25D_3 contralateral control; $ $p < 0.05$ vs. vehicle treatment.

GRO levels were increased in vehicle treated group at day 28 compared to day 1. Treatment of 24R,25$(OH)_2D_3$ decreased GRO to day 1 levels (Figure 4.11A). MIP1α and MIP3α levels were higher in vehicle treated group at day 28 compared to both vehicle and 24R,25$(OH)_2D_3$ treated groups at day 1. 24R,25$(OH)_2D_3$ treatment decreased levels of MIP1α and had no effect on MIP3α (Figure 4.11B, 4.11C). MCP-1 levels were lower in 24R,25$(OH)_2D_3$ treated rats compared to vehicle control at day. Vehicle treated rats at day 28 had lower levels of MCP-1 compared to its levels at day 1. 24R,25$(OH)_2D_3$ decreased levels of MCP-1 at day 28 compared to the vehicle treated group (Figure 4.11D). RANTES levels were higher at day 28 in

the vehicle treated group compared to both groups at day 1. 24R,25$(OH)_2D_3$ treatment decreased the levels of MCP-1 at day 28 to day 1 levels (Figure 4.11E).

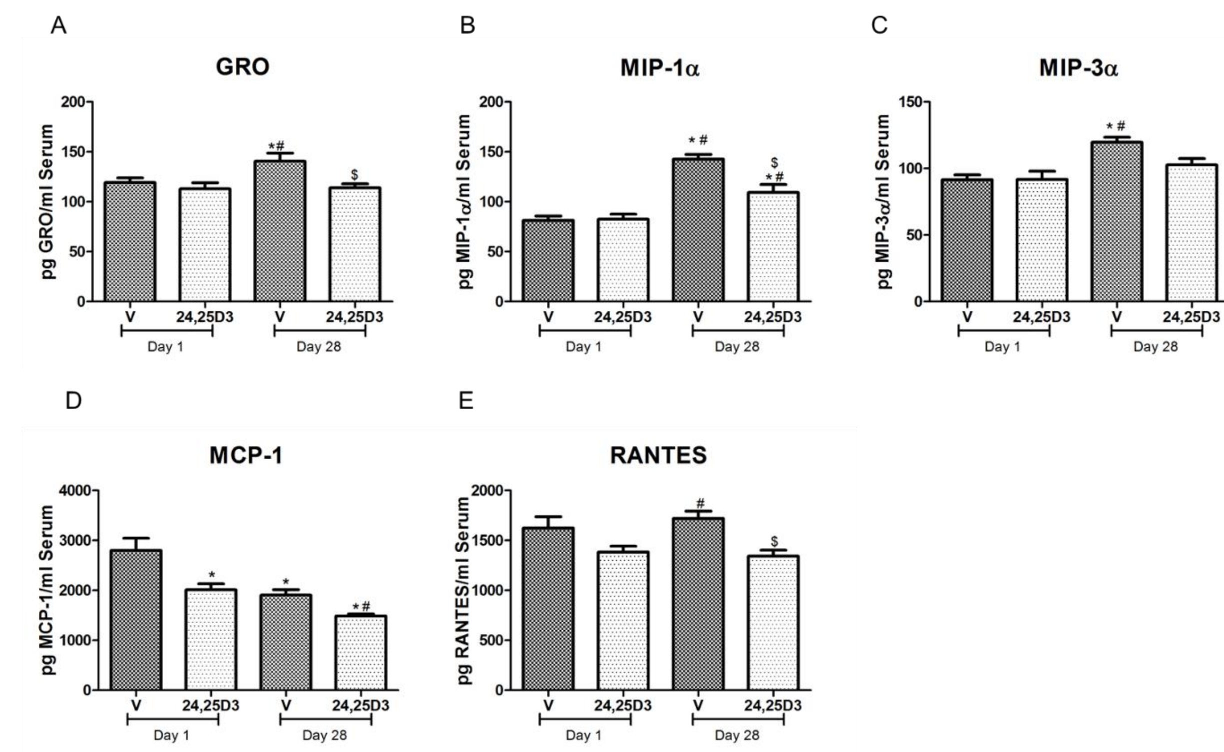

Figure 4.11: Serum levels of chemokines. * $p < 0.05$ vs. vehicle treated day 1; # $p < 0.05$ vs. 24,25D_3 treated day 1; $ $p < 0.05$ vs. vehicle treated day 28.

There were no significant differences in levels of IL-1α, IL-1β, IL-2, IL-6 and IL-7 between vehicle and 24R,25$(OH)_2D_3$ treated groups at day 1. At day 28, levels of these pro-inflammatory cytokines were higher in vehicle treated group compared to levels of both groups at day 1 (Figure 4.12A, 4.12B, 4.12C, 4.12D and 4.12E). Treatment of 24R,25$(OH)_2D_3$ lowered the levels of these cytokines.

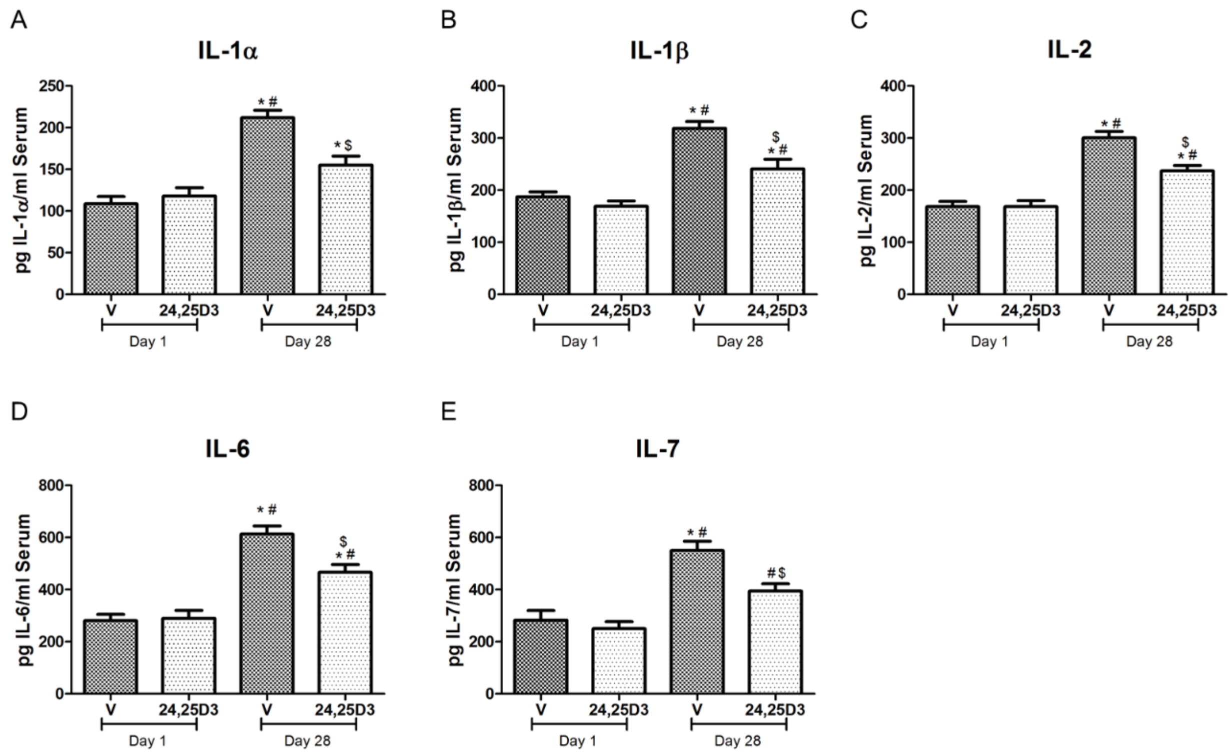

Figure 4.12: Serum levels of pro-inflammatory cytokines. * $p < 0.05$ vs. vehicle treated day 1; # $p < 0.05$ vs. 24,25D_3 treated day 1; $ $p < 0.05$ vs. vehicle treated day 28.

There were no significant differences in levels of IL-12, IL-17 and IL-18, IFN-g and TNF-α between vehicle and 24R,25$(OH)_2D_3$ treated groups at day 1. At day 28, levels of these inflammatory factors were higher in vehicle treated group compared to levels of both groups at day 1 (.13A, 4.13B, 4.13C, 4.13D and 4.13E). Treatment of 24R,25$(OH)_2D_3$ lowered the levels of these cytokines.

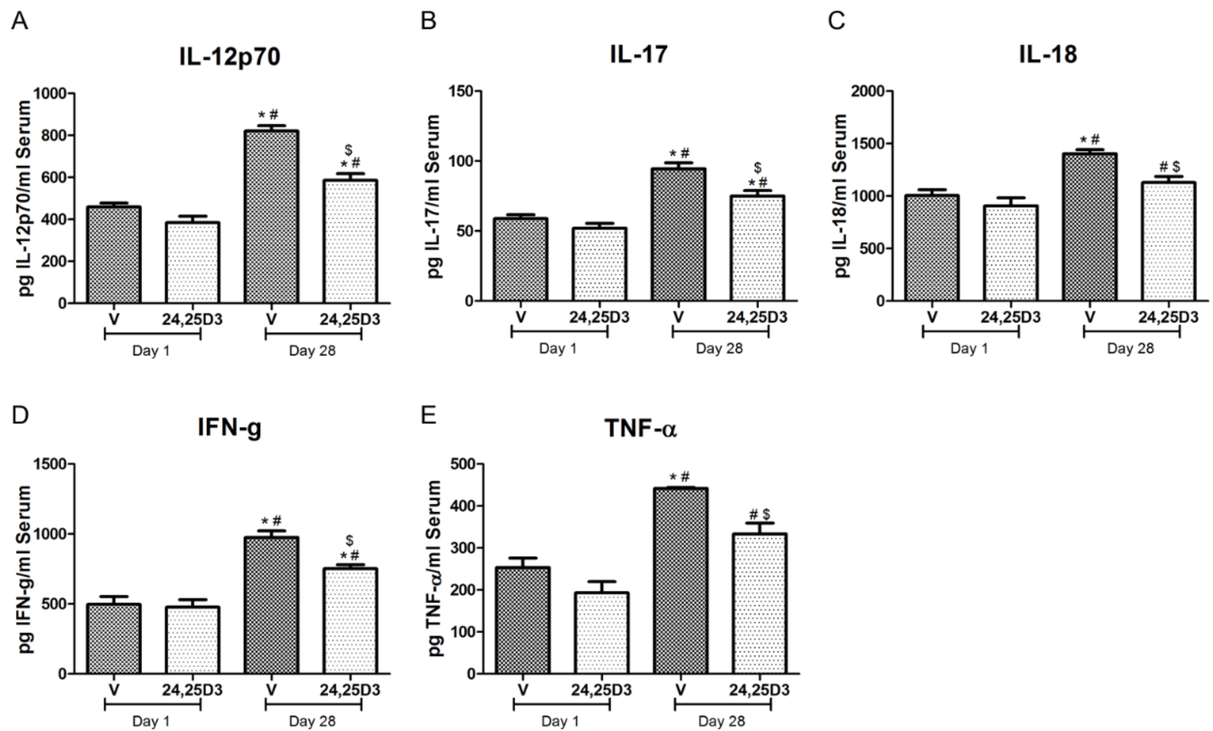

Figure 4.13: Serum levels of inflammatory factors. * $p < 0.05$ vs. vehicle treated day 1; # $p < 0.05$ vs. $24,25D_3$ treated day 1; $ $p < 0.05$ vs. vehicle treated day 28.

There were no significant differences in levels of IL-4, IL-5 and IL-10, IL-13 and M-CSF between vehicle and $24R,25(OH)_2D_3$ treated groups at day 1. At day 28, levels of IL-4 and IL-5 stayed the same in the vehicle treated group. $24R,25(OH)_2D_3$ significantly increased levels of IL-4 and IL-5 compared to vehicle treated at day 28 and compared to both groups at day 1 Figure 4.14A and 4.14B). IL-10 levels in vehicle treated group increased compared to day 1 and $24R,25(OH)_2D_3$ treatment further enhanced this increase (Figure 4.14C). Both vehicle and $24R,25(OH)_2D_3$ treated groups had higher levels of IL-13 at day 28 compared to day 1 and there were no significant differences between the two treatments (Figure 4.14D). M-CSF levels decreased in the vehicle treated group at day 28 compared to both vehicle and $24R,25(OH)_2D_3$

treated groups at day 1 and treatment of $24R,25(OH)_2D_3$ restored M-CSF levels back to day 1 levels (Figure 4.14E).

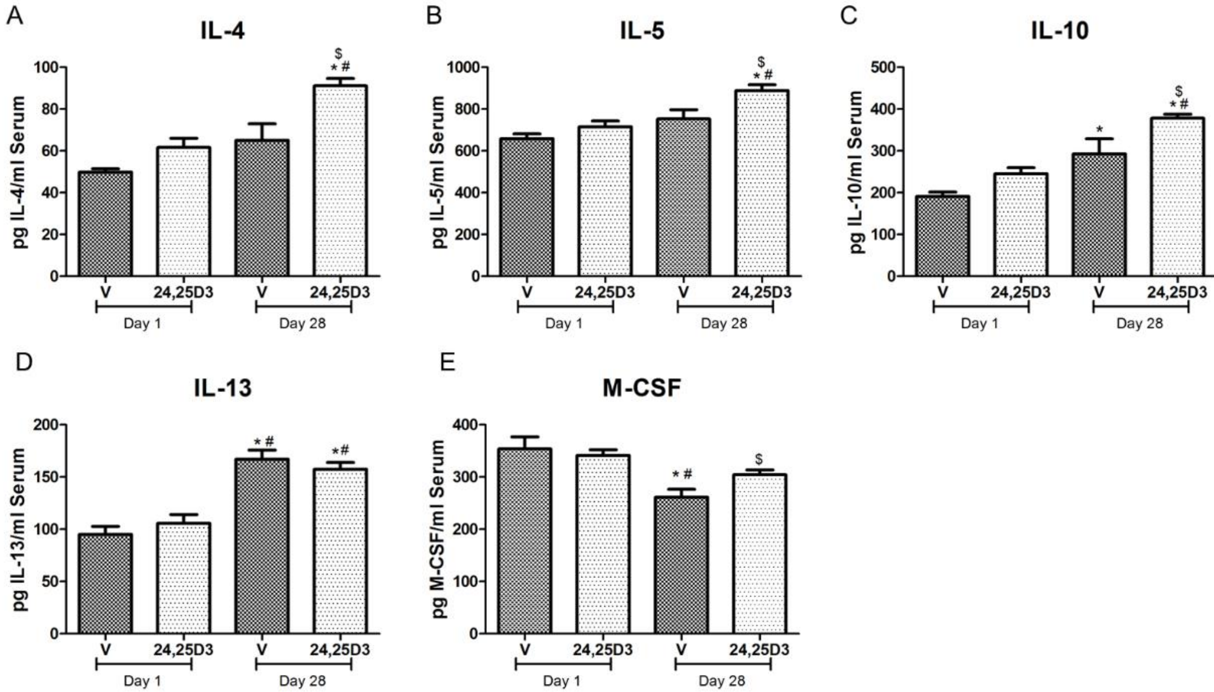

Figure 4.14: Serum levels of anti-inflammatory factors. * $p < 0.05$ vs. vehicle treated day 1; # $p < 0.05$ vs. $24,25D_3$ treated day 1; $ $p < 0.05$ vs. vehicle treated day 28.

Transforming growth factor β1 (TGF-β1) levels were higher in $24R,25(OH)_2D_3$ treated group compared to vehicle treated at day 1. This trend of increase continued to day 28 (Figure 4.15A). There were no significant differences in day 1 and day 28 levels of TGF-β2 and TGF-β3. In addition, there were no significant differences between vehicle and $24R,25(OH)_2D_3$ treatments at both time points (Figure 4.15B and 4.15C). VEGF levels were higher in both treatment groups at day 28 compared to day 1. Treatment of $24R,25(OH)_2D_3$ reduced this increase in VEGF levels

to day 1 levels (Figure 4.15D). EPO levels were higher in both treatment groups at day 28 compared to day 1. Furthermore, treatment of 24R,25$(OH)_2D_3$ compared to vehicle treated group (Figure 4.15E).

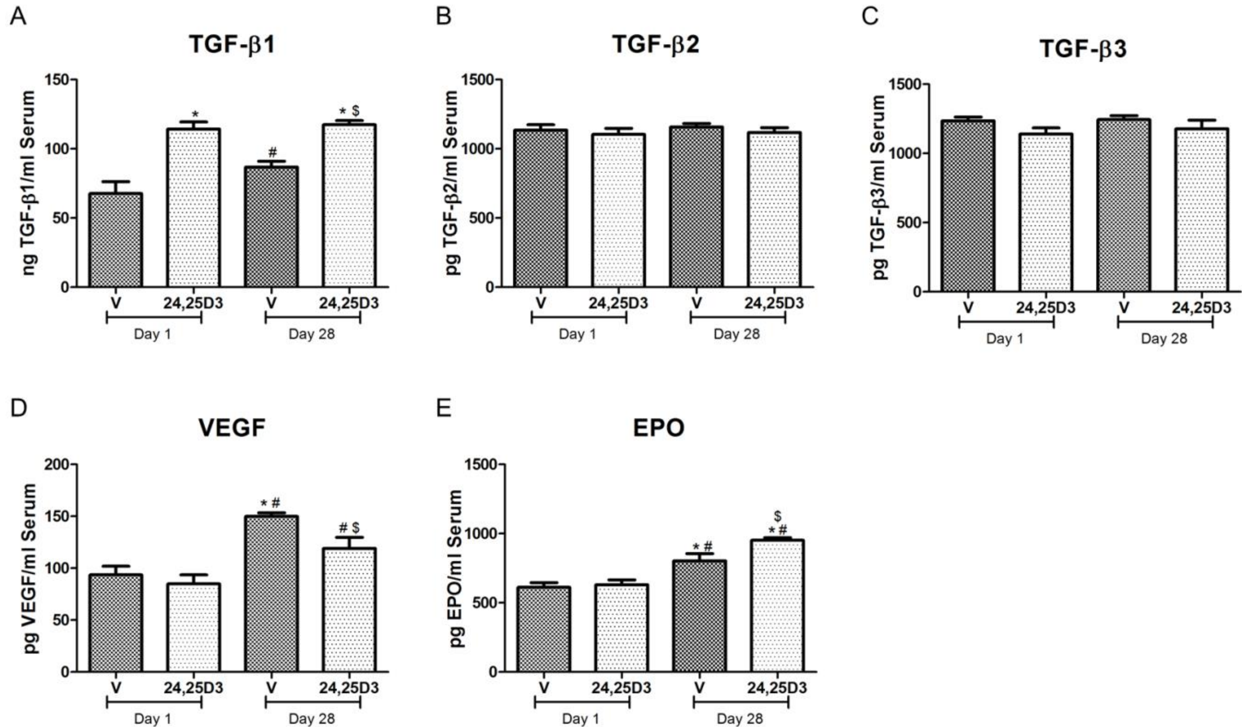

Figure 4.15: Serum levels of growth factors protein. * $p < 0.05$ vs. vehicle treated day 1; # $p < 0.05$ vs. 24,25D_3 treated day 1; \$ $p < 0.05$ vs. vehicle treated day 28.

DISCUSSION

Translational research provides insights into mitigating the long-term effects of OA disease process. Animal models are widely used to investigate effective of treatment of knee osteoarthritis. Smaller animals are useful because of their ease of use and cost. In our study, we chose to use a rat model for preliminary testing as their cartilage have been shown to be able to induce both partial and full-thickness cartilage defects [54] . OA was induced in the study through ACLT. The ACLT model has been shown to demonstrate similar symptoms as clinical

OA, with animals developing a more localized defect compared to other models [129, 147].

Normal and osteoarthritic articular cartilage functions can be assessed quantitatively in term of morphological properties (thickness, volume, joint surface area), and biomechanical properties (equilibrium modulus, dynamic stiffness and hydraulic permeability) as well as biological properties (composition, cell proliferation and apoptosis, protein production). In our study, ACLT induced OA like changes as results from the study show both morphological and biological changes in knees treated with vehicle compared to controls. Composite scores for the OA knees were around 2-3, which is supported by literature findings that animals underwent ACLT for four weeks scored around 4-5 for histopathology [47].

In our study, 24R,25$(OH)_2D_3$ treatment reduced histopathological scores in induced OA knees, suggesting that 24R,25$(OH)_2D_3$ restored cartilage loss. Not only there were more volume of cartilage, there were also more proteoglycan stained. 24R,25$(OH)_2D_3$ has been shown to play important roles in cartilage development, chondrocyte proliferation, differentiation and inhibiting growth plate chondrocyte apoptosis [10, 18, 34, 65]. These results suggest that 24R,25$(OH)_2D_3$'s protective effect could have been through inhibiting articular chondrocyte apoptosis and promoting synthesis of new ECM.

Our results showed increased levels of pro-inflammatory cytokines, chemokines and macrophage stimulating factors in the induced OA synovial fluids compared to controls. The elevation in these factors in the presence of moderate cartilage degradation is supported by studies showing that the systemic inflammation observed in OA is at least partially reflective of local synovial inflammation [114, 132]. In addition, recent observations indicate that inflammation is present in OA joints well before the development of significant radiographic change [5]. Furthermore, it has been shown that are increased mononuclear cell infiltration and

overexpression of inflammatory mediators in early stage of the disease [8], which corroborate our findings. A study comparing inflammatory cytokines profiles associated with osteoarthritis and rheumatoid arthritis demonstrate that similar cytokine profiles are present in the synovial fluid of both diseases, further supporting that inflammation plays important roles in osteoarthritis.

Extracellular matrix breakdown is ubiquitous in OA cartilage degeneration. It has been shown that osteoarthritic cartilage can activate inflammatory cascade and immune response [156]. Our results show that increased levels of pro-inflammatory cytokines, chemokines and other inflammatory mediators were found in joints with induced OA compared to the control. These results are supported by findings that show activation of membrane attack complex leads to release of chemokines and cytokines into the joint space and further exacerbate the pathogenic response in OA and lead to cartilage degradation [156].

Our results show that levels of pro-inflammatory mediators were decreased and increased levels of anti-inflammatory factors were increased in the $24R,25(OH)_2D_3$ treated rats. These results suggest that a possible mechanism of $24R,25(OH)_2D_3$ in reducing cartilage degeneration in OA could be through mediating chronic low inflammatory conditions during diseases progression. Currently, most of the anti-inflammatory drugs used to alleviate OA associated pain and inflammation do not have disease modifying properties.

CONCLUSION

Biomechanical properties of the articular cartilage depend on the biochemical compositions of the cartilage and the interaction between matrix molecules. Our results indicate that $24R,25(OH)_2D_3$ reduced pathological changes of OA. Taken together with our in vitro

studies showing anti-apoptotic and anti-inflammatory effects of $24R,25(OH)_2D_3$, this study may suggest $24R,25(OH)_2D_3$ could be used to halt progression of knee OA.

CHAPTER 5

CHARACTERIZATION OF HUMAN OSTEOARTHRITIC KNEES INDICATE POTENTIAL SEX DIFFERENCES

SUMMARY

Many musculoskeletal conditions are affected by the sex of the patient. The prevalence of osteoarthritis (OA) is higher among women than among men in every age group and with age, the overall prevalence of OA increases. Exploring the potential for underlying sex-specific differences in the physiological and biochemical properties of osteoarthritic joint tissues may permit the development of sex-specific therapies. The vitamin D_3 metabolite $1\alpha,25(OH)_2D_3$ and sex hormones have been shown to regulate cartilage and bone development and homeostasis in a sex dependent manner, which suggests that genetic sex plays an important role in regulating OA joint biology. To begin to examine this, knee joints of 10 male and 10 female Caucasian patients 65-75 years of age were obtained during total joint replacement. Levels of vitamin D_3 metabolites, cytokines, matrix metalloproteinases, and growth factors in the synovial fluid were determined**.** Expression of receptors for $1\alpha,25(OH)_2D_3$ and estrogen in primary cultures of **c**hondrocytes isolated from articular cartilage adjacent to the OA lesions and osteoblasts in the subchondral was determined. Chondrocytes and osteoblasts were treated with 10^{-8} M $1\alpha,25(OH)_2D_3$, 17β-estradiol (E2), and dihydrotestosterone (DHT) and effects on gene expression assessed. Sex differences were found in basal levels of hormone receptors, synovial fluid levels of vitamin D metabolites, cytokines, and metalloproteinases. Male cells were more responsive to $1\alpha,25(OH)_2D_3$ and DHT. E2 reduced OA changes in female cells, suggesting hormonal modulation could contribute to the sex differences in severity and development of knee

OA. This study is the first to demonstrate sex differences in tissues from osteoarthritic knees. Results from this study have implications for potentially different preventions and treatments of OA between male and female patients. Further research is needed to better understand these sex-based differences.

INTRODUCTION

Osteoarthritis (OA) is a leading cause of pain and disability in adults. Nearly one in two people may develop symptomatic knee OA by age 85 [98]. OA affects the whole joint, i.e., the cartilage, bone, muscle, tendons and synovium and impacts daily life and function. Symptoms may include joint pain, tenderness, stiffness, and locking [150]. A variety of causes including hereditary, developmental, metabolic, or mechanical deficits may initiate processes leading to cartilage loss. Risk factors for OA include gender, race/ethnicity, joint instability, obesity, joint trauma, and age.

Epidemiological studies show sex-specific differences in prevalence and severity of OA [119, 160]. On average, the rate of arthritis is 58% higher in women than in men [145]. Women report more pain than men due to OA and are more likely to have reductions in function and quality of life [130]. Biomechanical differences between men and women may contribute to differences in OA prevalence, although data supporting this are not clear [103]. Thus, other sex differences may be involved.

Studies indicate that bone and cartilage cells exhibit sex-specific responses to the vitamin D metabolites, 1α,25-dihydroxyvitamin D3 [$1,25(OH)_2D_3$] and $24,25(OH)_2D_3$ [107, 111, 125], suggesting that sex differences in response to vitamin D metabolites may affect the development and progression of OA. In addition to its role in maintaining calcium homeostasis and bone metabolism [13, 110, 135], recent evidence suggests that $1,25(OH)_2D_3$ plays important roles in

cartilage remodeling and participates in the inflammatory response [60, 136]. Epidemiological studies have correlated low serum levels of 25-hydroxyvitamin D_3 [$25(OH)D_3$], the precursor of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ with OA progression [41]. Moreover, vitamin D supplementation had a protective effect against OA in rats by reducing mRNAs for MMP-3, IL-1β, and TNF-α, factors that are associated with cartilage degradation and inflammation [23]. Interestingly, $1\alpha,25(OH)_2D_3$ induced production of 17β-estradiol in female rat chondrocytes, but not male chondrocytes, further demonstrating that there is sexual dimorphism at the cellular level [141].

Sex hormones are important regulators of cartilage biology. Ovariectomized cynomolgus monkeys exhibit histopathological features typical of OA, suggesting that estrogen has a protective effect [56]. Studies using a mouse model of OA support this; ovariectomized female mice had more OA than intact mice but orchidectomized male mice had less severe OA than intact males [90]. A number of studies have shown sexual dimorphism in the response of chondrocytes and osteoblasts to 17β-estradiol (E2) and dihydrotestosterone (DHT) at the cellular level [38, 39, 102, 142]. However, the effects of sex hormones on the incidence and progression of knee OA, as well as on the regenerative potential of affected cartilage are poorly understood.

Dysregulation of Wnt signaling pathways may play a role in the development of OA with the potential for sex differences in this dysregulation. Because Wnt pathways are involved in both cartilage and bone formation and regulation of chondrocyte hypertrophy in the growth plate, dysregulation of Wnt pathways in adult tissues could contribute to the chondrocyte hypertrophy seen in OA. DKKs1-4, Wnt antagonists, were reported to be present in articular cartilage with DKK-1 also found in human OA synovium [158]. Studies have shown that $1\alpha,25(OH)_2D_3$ and

Wnt5a mediate their effects via similar receptor components and suggests that these two pathways may interact [36].

The aim of this study was to determine if sex-specific differences are present in tissues from males and females that have developed significant knee OA. Clinically, all patients receiving total joint replacement exhibited comparable levels of OA severity, eliminating disease progression as a variable. We focused on potential differences in synovial fluid factors, serum $25(OH)D_3$, presence of receptors for $1,25(OH)_2D_3$ and 17β-estradiol in isolated chondrocytes and osteoblasts, and the response of these cells to $1\alpha,25(OH)_2D_3$, $24R,25(OH)_2D_3$, E2 and DHT.

MATERIALS AND METHODS

Study Subjects and Data Collection

Power analysis determined that twenty patients, 10 male and 10 female were needed in order to detect statistically relevant differences. Twenty white, non-Hispanic patients (10 males and 10 females) between the ages of 65 and 75 that were undergoing joint replacement surgery due to significant osteoarthritis were included in this study. Patients with any of the following characteristics were excluded: inflammatory arthritis, osteonecrosis, prior upper tibial osteotomy, premenopausal women, age less than 65 years or age greater than 75 years, insulin dependent or diabetic, BMI greater than 30, or history of knee infection.

Information collected at the Mayo Clinic prior to surgery included demographics (age, sex, BMI); medications and supplements (vitamin D supplements, bisphosphonates use, and estrogen use); SF-12, WOMAC, and PASE functional scale tests; serum $25(OH)D_3$; and standard radiographs. Within two weeks prior to surgery, patients completed the OARSI-OMERACT pain scale [58], the knee pain map [146], and pressure pain thresholds at the knee using JTECH (wireless) version 5 software [118, 154].

Fresh human tissue was obtained from consenting patients undergoing elective total knee arthroplasty at Mayo Clinic, Jacksonville, FL. The fluid and tissue obtained are normally discarded during the course of the procedure. Synovial fluid was aspirated from the knee joint prior to skin incision, snap frozen and stored at -80°C. Bone with overlaying cartilage specimens were from the distal and posterior medial and lateral femoral condyles and the proximal tibial bone cut. Bone/cartilage specimens were shipped on ice to the Georgia Institute of Technology (Atlanta, GA) or to the School of Engineering Institute for Engineering and Medicine at Virginia Commonwealth University (VCU, Richmond, VA). Upon receipt, the knee tissues were dissected, separating articular cartilage, subchondral bone, synovial membrane, and meniscus. Tissues were coded using the de-identified patient number provided by Mayo. Articulating surfaces of the tibia and femur were photographed. Chondrocytes and osteoblasts were isolated at time of receipt; meniscus, synovium, bone and cartilage were processed for histology as described below. Synovial fluid was thawed and aliquoted, then stored at -80°C until used. All assays performed at VCU were blinded to donor sex.

Histology of Knee Tissues

Lateral and medial menisci were dehydrated in a series of 95% and 100% ethanol and xylene washes, then the samples were embedded in paraffin. Sections 7-µm thick were stained with haematoxylin and eosin (H&E). Synovium samples were embedded in OCT compound and then were cut into 7-µm sections using a cryostat. Synovium samples were stored in -80◦C until they were stained. Sections were imaged using a Zeiss Observer Z1 microscope using a 20x objective.

Following fixation in 10% formalin, cartilage and bone samples were decalcified (Decal Chemical Corporation, Tallman, NY, USA) for 16 hours on a rotating platform before being

dehydrated in a series of 95% and 100% ethanol and xylene washes. Samples were embedded in paraffin; 7-µm sections were stained with H&E. Cartilage tissues were also stained with safranin-O to assess glycosaminoglycan content.

Synovial Fluid

Synovial fluid levels of $1,25(OH)_2D_3$ were measured according to manufacturer's instructions using a human $1,25(OH)_2D_3$ ($DHVD_3$) ELISA Kit (Novatein Biosciences, Cambridge, MA, USA). Levels of $25(OH)D_3$ were measured according to manufacturer's instructions using a human 25-Hydroxyvitamin D_3 ($25HVD_3$) ELISA Kit (Novatein Biosciences). In addition, we used an ELISA kit from Immunodiagnostic Systems (Gaithersburg, MD, USA) that measured $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$. We then approximated the amount of $24,25(OH)_2D_3$ plus $25(OH)D_2$ by subtracting the values for $25(OH)D_3$ that were obtained using the Novatein Biosciences assay. Because other vitamin D metabolites might also be present, including $1,24,25(OH)_3D_3$, mass spectrometry (Thermo Vantage Triple Quadrupole Mass Spectrometer, Biotrial, Quebec, Canada) was used to measure $24,25(OH)_2D_3$ specifically. Data were normalized to synovial fluid volume.

Inflammatory cytokines and matrix metalloproteinases present in the synovial fluid were measured using a Luminex screening assay (R&D Systems, Minneapolis, MN, USA). This assay uses superparamagnetic beads coated with analyte-specific antibodies. TGF-β1, 2, and 3 levels were measured using a magnetic bead–based multiplex assay (Bio-Rad, Hercules, CA, USA). Sulfated glycosaminoglycan levels were measured using the dimethyl methylene blue assay (Sigma Aldrich, St. Louis, MO, USA) [48]. In all cases, data were normalized to synovial fluid volume.

Isolation of Chondrocytes and Osteoblasts

Articular cartilage from areas of minimal fibrillation and from areas of maximum erosion was obtained by sharp dissection for each patient (Figure 1C). Cartilage pieces were minced and incubated in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher, Waltham, MA, USA) containing 0.25% trypsin (Life Technologies, Carlsbad, CA, USA) for 30 minutes at 37°C. After discarding the trypsin digest, chondrocytes were isolated from cleaned cartilage fragments by incubating them for 16 hours in 0.03% collagenase type II (Worthington Biosciences, Lakewood, NJ) in DMEM containing 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were separated from remaining cartilage pieces by straining the mixture through a 40-µm filter. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and 100 U/mL penicillin and 100 µg/mL streptomycin. Passage 1 cells were used for all experiments and cells were seeded at 15,000 per cm^2 for all experiments.

Subchondral bone samples obtained from regions under minimally fibrillated cartilage and under regions of maximal cartilage erosion were minced into small chips, incubated in 0.25% trypsin for 30 minutes at 37°C, and then washed with DMEM with 1% penicillin-streptomycin three times. After washing, bone chips were cultured for two weeks in Petri dishes containing DMEM with 1% penicillin-streptomycin and 10% FBS to enable osteoprogenitor cells to migrate out onto the dish surface. Media were changed once a week until cells reached confluence. Cells were then subcultured by seeding at 10,000 cells per cm^2 for all experiments.

Characterization of Chondrocytes

First passage cells from six patients of each sex were used for these experiments. In order to determine if chondrocytes were competent to respond to hormone treatment, mRNA levels for receptors for $1,25(OH)_2D_3$ (nVDR and PDIA3) or 17β-estradiol (ERα66 [ESR1] and

ERα36) were measured in chondrocytes and osteoblasts from both fibrillated and minimally affected OA tissues via real-time qPCR [25]. The cells were further characterized with respect to basal expression of inflammatory cytokines (IL1A, IL1B, IL6, IL8, IL10) [66]. Related work has shown that differentiation of chondrocytes in response to $1,25(OH)_2D_3$ involves signaling via wingless-Int pathway molecules (WNT3A, WNT5A, CTNNB, DKK1, and DKK2) [108]. Therefore, we also measured RNA expression for these proteins. Primers for each of these mRNAs are shown in Supplemental Table 1. QuantiTect primers were purchased from Qiagen (Valencia, CA, USA).

RNA was harvested using a TRIzol® (Invitrogen, Carlsbad, CA, USA) extraction method following the manufacturer's protocol. RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and specific mRNAs (250 ng) were amplified using reverse transcription (High Capacity cDNA Reverse Transcription kit, Life Technologies). Starting quantities of mRNA were determined using SybrGreen chemistry (Power SYBR® Green PCR Master Mix, Life Technologies) in a StepOne Plus imaging system (Life Technologies). All gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In order to examine if there are sex differences in response to hormonal treatment, confluent first passage chondrocytes were treated with 10^{-8} M $1\alpha,25(OH)_2D_3$ (Enzo Life Sciences, Plymouth, PA, USA) or 10^{-8} M 17β-estradiol (Sigma Aldrich, St. Louis, MO, USA). In addition, cells were treated with 10^{-7} M $24R,25(OH)_2D_3$ (Enzo Lifesciences) or 10^{-8} M 5α-dihydrotestosterone (DHT) (Sigma Aldrich).

Expression of chondrocyte genes was assayed at 12 hours. mRNAs for aggrecan (ACAN), type II collagen (COL2A1), and cartilage oligomeric matrix protein (COMP) was

measured using real-time qPCR as described above. Primers used are listed in supplemental table 1.

To measure alkaline phosphatase specific activity, confluent first passage cells were treated for 24 hours with either vehicle (0.01% ethanol), or 10^{-8} M $1\alpha,25(OH)_2D_3$ or E2. Cells were harvested by trypsin digestion and were lysed in Triton X-100 (Sigma Aldrich). Alkaline phosphatase specific activity was measured by assaying the release of *p*-nitrophenol from *p*-nitrophenylphosphate at a pH of 10.2 in the cell lysates. Enzyme activity was normalized to total protein content of the cell lysates (BCA Protein Assay; Thermo Fisher Scientific, Waltham, MA, USA) [155].

Osteoblasts

Confluent first passage osteoblasts were treated with either vehicle (0.01% ethanol) or 10^{-8} M $1\alpha,25(OH)_2D_3$, E2 or DHT for 24 hours. The conditioned media were collected and levels of secreted osteocalcin and osteoprotegerin were determined. Osteocalcin was measured using a commercially available radioimmunoassay following manufacturer's instructions (Biomedical Technologies, Stoughton, MA, USA) [106]. Osteoprotegerin was measured by ELISA (R&D Systems, Minneapolis, MN, USA) [109]. After decanting the conditioned media, cells were harvested by trypsin digestion and alkaline phosphatase specific activity was determined in cell lysates as described above. DNA was measured using a fluorometric assay from Promega (Madison, WI, USA). Levels of secreted factors in the conditioned media were normalized to total DNA.

Statistical Analysis

Clinical data were summarized for each sex by the sample median and interquartile range for numerical variables while categorical variables were summarized by frequency and

percentage. Comparisons between males and females were evaluated using a Wilcoxon rank sum test for numerical variables, Cochran-Armitage test for trend for ordered variables, and Fisher's exact test for categorical variables. p-values < 0.05 are considered statistically significant without adjustment for multiple testing owing to the exploratory and hypothesis-generating nature of the study. The considerable number of tests being performed increases the chance of a Type I error, and interpretation of the results should consider this. Additionally, the small sample size increases the chance of a Type II error (ie. false-negative association). Statistical analyses were performed using SAS statistical software (version 9.3; SAS Institute Inc.; Cary, NC).

Data from the cell study presented are the mean ± SEM of n=6 independent cultures for each patient per variable. Six patients of each sex were examined for the cell hormone treatment study. Statistical significance was determined by ANOVA with post-hoc Bonferroni's modification of Student's t-test. For graphs labeled as treatment over control, the value of each sample from the treated group was divided by the mean of the control group. Each data point represents the mean ± SEM for six normalized values. The control is represented by a dashed line with a value equal to one. Significance was determined by Mann Whitney test. $p \leq 0.05$ was considered to be significant.

RESULTS

Patient Demographics and Clinical Findings

Patient demographics and clinical characteristics are summarized in Table 5.1 according to sex and statistical differences between sexes are presented. Of the 10 males and 10 females in the study, the median age was 69 years (range, 65 to 75 years) and median BMI was 26.5 (range, 21.0 to 29.0). Although not statistically significant, there was some evidence to suggest that previous or current vitamin D use was more common in females than males (90% vs. 50%,

p=0.14). As expected, previous or current estrogen use was more common in females than males (80% vs. 0%, p<0.001). There was no evidence of a difference in age, BMI, bisphosphonate use, prior knee surgery, prior intra-articular steroid injections, or prior intra-articular hyaluronic acid injections between males and females (all p≥0.21).

Table 5.1. Patient demographics and clinical history prior to total knee replacements

Variable	Female	Male	P-value [a]
Median age (IQR) - yrs	70 (68, 71)	69 (66, 72)	0.42
Median body mass index (IQR) – kg/m^2	26.2 (25.6, 28.1)	27.0 (24.3, 28.6)	0.34
Vitamin D use – no. (%) [b]			0.14
Currently	6 (60%)	3 (30%)	
Previously	3 (30%)	2 (20%)	
Never	1 (10%)	5 (50%)	
Median 25-hydroxy D3 (IQR) - ng/ml	42.1 (33.7, 47.2)	38.4 (31.0, 52.6)	0.79
Bisphosphonates use – no. (%) [b]			0.47
Previously	2 (20%)	0 (0%)	
Never	8 (80%)	10 (100%)	
Estrogen use – no. (%) [b]			<0.001
Currently	4 (40%)	0 (0%)	
Previously	4 (40%)	0 (0%)	
Never	2 (20%)	10 (100%)	
Previously injured a knee so badly it was difficult to walk for at least a week – no. (%)	0 (0%)	3 (30%)	0.21
Prior knee surgery – no. (%)	6 (60%)	6 (60%)	1
Prior intra-articular injections of steroids – no. (%)	7 (70%)	5 (50%)	0.65
Median no. of steroid injections (IQR)	1 (0, 1)[c]	1 (0, 3)	0.58
Prior intra-articular injection of hyaluronic acid – no. (%)	3 (30%)	5 (50%)	0.65
Median no. of hyaluronic injections (IQR)	0 (0, 1)	2 (0, 3)	0.3
Knee to be replaced – no. (%)			1
Right	6 (60%)	6 (60%)	
Left	4 (40%)	4 (40%)	

Abbreviations: IQR, interquartile range.
[a] P-values result from a Wilcoxon rank sum test for numerical variables and Fisher's exact test for categorical variables.
[b] P-values are based on categorization as currently or previously used vs. never used.
[c] Information was not available for 1 patient.

Preoperative radiographic and knee pain assessments are summarized in Table 5.2 according to sex. The pain pressure threshold 1 cm above the medial joint line was significantly

lower in females than males (median, 3.0 vs. 5.1 kg, p=0.007). There was no evidence of a difference in serum 25(OH)D_3, radiographic assessment, knee pain, or areas of knee pain (all $p \geq 0.37$). There was no evidence of a difference between males and females in the pain and functional assessment prior to surgery, including the WOMAC, OARSI-MERACT, and SF-12 (all $p \geq 0.36$, Table 5.3).

Table 5.2. Pre-operative knee pain and radiographic assessment

Radiographic Assessment	Female	Male	
Kellgren-Lawrence Grading Scale – no. (%)			0.37
Grade 3	1 (10%)	0 (0%)	
Grade 4	9 (90%)	10 (100%)	
Median pressure pain threshold (IQR) – kg			
Medial joint line	4.5 (3.7, 5.1)	5.3 (4.1, 6.9)	0.16
1 cm above the medial joint line	3.0 (2.7, 3.8)	5.1 (4.5, 6.2)	0.007
1 cm below the medial joint line	4.0 (3.5, 4.1)	5.6 (4.0, 6.8)	0.1
Area(s) of knee pain – no. (%) [b]			
Localized			
Superior lateral	0 (0%)	1 (10%)	1
Patella	0 (0%)	1 (10%)	1
Medial joint line	3 (30%)	2 (20%)	1
Inferior lateral	1 (10%)	0 (0%)	1
Inferior medial	1 (10%)	0 (0%)	1
Regional			
Medial	1 (10%)	1 (10%)	1
Patella	1 (10%)	2 (20%)	1
Diffuse pain	4 (40%)	3 (30%)	1

[a] P-values result from a Wilcoxon rank sum test for numerical variables and Fisher's exact test for categorical variables.

[b] More than one location per patient was possible. Pain was not reported at any of the following locations: superior medial, lateral joint line, back of knee, lateral (regional), and back of knee (regional).

Table 5.3. Pain and functional assessment prior to surgery

Variable	Female	Male	P-value [a]
WOMAC			
Median total raw score (IQR)	39 (32, 47)	45 (27, 55)	0.76
Median pain score (IQR)	8.5 (7, 10)	9.0 (5, 12)	0.73
Median stiffness score (IQR)	4.0 (2, 5)	4.0 (3, 6)	0.64
Median difficulty performing daily activities score (IQR)	28 (21, 33)	32 (18, 38)	0.7
OARSI-OMERACT pain scale			
Median total pain score (IQR)	49 (30, 59)	42 (39, 61)	0.88
Median constant pain subscore (IQR)	45 (20, 55)	38 (25, 45)	0.36
Median intermittent pain subscore (IQR)	48 (42, 63)	50 (42, 67)	0.7
SF-12			
Median physical scale (IQR)	33 (29, 42)[b]	38 (30, 39)	0.55
Median mental scale (IQR)	61 (54, 63)[b]	57 (54, 66)	0.9

[a] P-values result from a Wilcoxon rank sum test.
[b] Information was not available for 1 patient.

Tissue Morphology and Histology

All patients exhibited similar morphological and histological features. All patients had radiographic evidence of joint narrowing and bone-bone contact (Figure 5.1A). Figure 5.1B shows a healthy joint with no cartilage fibrillation. There was significant fibrillation of the cartilage (Figure 5.1C) and safranin-O staining indicated that there was loss of proteoglycans (Figure 5.1D). Histology of the synovium showed hyperplasia and infiltration of adipocytes (Figure 5.1E). The meniscus appeared to have dense intra-articular aggregates of inflammatory

cells (Figure 5.1F).

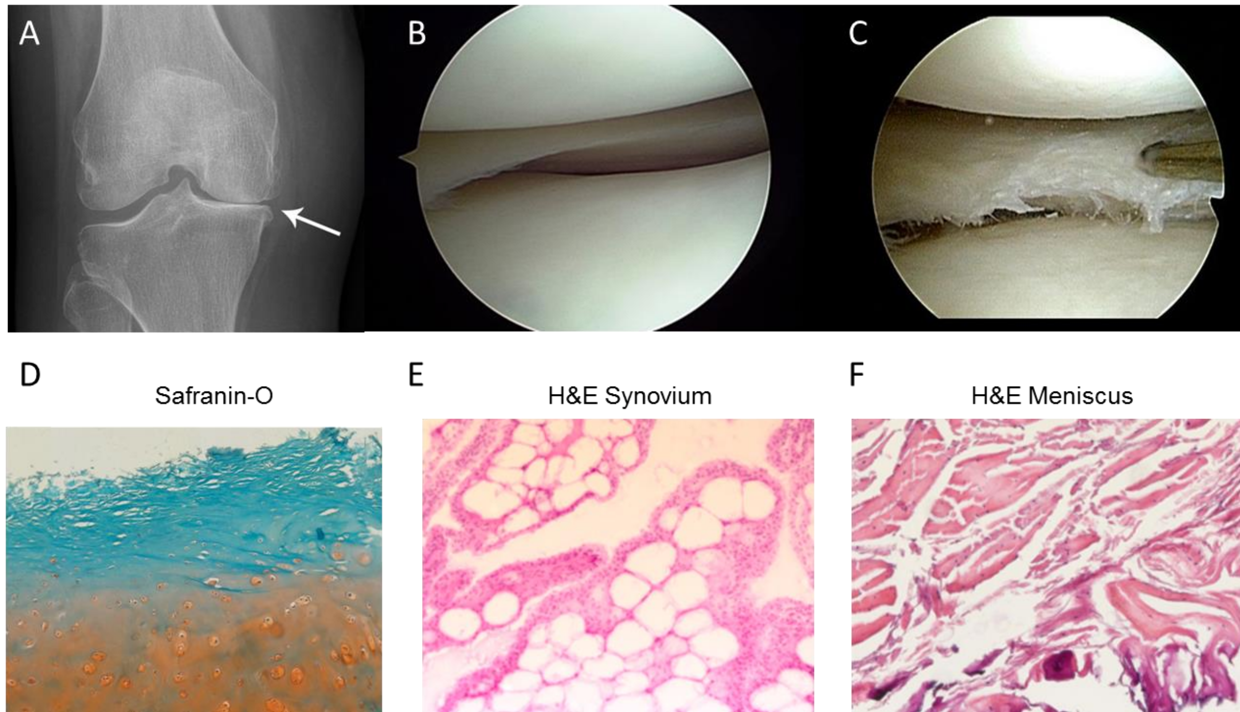

Figure 5.1: X-Ray image showing joint narrowing and bone-bone contact (A). Arthroscopic images of normal knee cartilage (B) and segment of bone and articular cartilage from routine bone resection during total knee arthroplasty. Articular cartilage from areas of minimal fibrillation (arrow) and from areas of maximum erosion (star) were obtained for each patient. (C). Safranin-O stained fibrillated cartilage showing proteoglycan (D). H&E stained synovium (E) and meniscus (F) isolated from OA knees.

Synovial Fluid

Synovial fluid differed between males and females (Table 5.4). Males had increased levels of MMPs 1, 7, 9 and 13. Hepatocyte growth factor (HGF), stem cell factor (SCF), and stem cell growth factor beta (SCGF-beta) were all higher in male synovial fluid than in females. The amounts of sulfated glycosaminoglycans (sGAG), TGF-β1 and TGF-β2 were higher in males compared to females. Females had higher levels of inflammatory cytokines, IL-2α, IL-3, IL-12p40, IL-16, IL18, TNF-β, and chondrocyte apoptosis inducing factor (TRAIL) compared to males. Higher levels of macrophage stimulators (leukemia inhibitory factor [LIF], macrophage colony-stimulating factor [M-CSF], macrophage migration inhibitory factor [MIF]), and pro-

inflammatory mediators (growth-regulated oncogene α [GRO-α], monocyte chemotactic protein-3 [MCP-3], and monokine induced by gamma interferon [MIG]) were found in females compared to males.

Table 5.4. Synovial Fluid

	Mean Value (pg/ml) ± SEM			
	Male	Female	p Value	
HGF	1075.00 ± 254.20	514.30 ± 46.10	0.0352	Males > Females
IFN-α2	279.30 ± 50.09	159.80 ± 12.10	0.0207	
IL-1α	389.00 ± 23.91	337.40 ± 9.80	0.0367	
MMP-1	2219.00 ± 539.60	1270.00 ± 84.74	0.049	
MMP-2	1981.00 ± 50.44	1811.00 ± 42.84	0.0248	
MMP-7	1189.00 ± 15.21	1147.00 ± 7.63	0.0173	
MMP-9	1565.00 ± 473.20	585.60 ± 135.50	0.0328	
MMP-12	39.20 ± 0.87	36.90 ± 0.46	0.0227	
MMP-13	441.90 ± 18.22	396.00 ± 7.241	0.0187	
SCF	478.00 ± 116.2	239.80 ± 21.45	0.0487	
SCGF-β	5503.00 ± 1713.20	1928.00 ± 433.60	0.0487	
TGF-β1	1723.00 ± 282.00	1070.00 ± 109.00	0.0464	
TGF-β2	35.79 ± 4.72	23.69 ± 2.74	0.0415	
sGAG	14.04 ± 2.02	6.25 ± 0.57	0.004	
GRO-α	459.40 ± 95.18	739.80 ± 61.36	0.0217	Females > Males
IL-2α	843.90 ± 166.50	1321.00 ± 120.20	0.0306	
IL-3	806.90 ± 204.80	1289.00 ± 55.29	0.0395	
IL-12p40	380.60 ± 81.14	578.20 ± 26.41	0.0342	
IL-16	708.10 ± 148.50	1353.00 ± 247.00	0.0449	
LIF	309.60 ± 60.31	480.80 ± 41.60	0.0294	
MCP-3	147.70 ± 29.88	220.10 ± 17.42	0.0466	
M-CSF	748.80 ± 161.60	1156.00 ± 107.00	0.0468	
MIF	752.60 ± 172.70	1665.00 ± 357.50	0.0323	
MIG	1793.00 ± 321.80	4252.00 ± 1018.00	0.0371	
TNF-β	148.00 ± 42.95	252.20 ± 8.99	0.0304	
TRAIL	714.90 ± 156.20	1100.00 ± 79.97	0.0332	
β-NGF	5.67 ± 2.76	3.42 ± 0.83	0.4791	Not significant
CTACK	613.00 ± 112.90	925.30 ± 172.90	0.1621	
IL-18	156.80 ± 8.56	160.90 ± 7.90	0.727	
IL-1β	214.70 ± 99.25	128.80 ± 13.42	0.4113	
MMP-3	2898.00 ± 81.25	2961.00 ± 100.4	0.6544	
MMP-8	1183.00 ± 310.20	985.50 ± 103.40	0.4934	
SDF-1α	506.50 ± 79.08	461.70 ± 51.30	0.6259	

No sex-specific differences in the concentration of $1,25(OH)_2D_3$ in the synovial fluid were detected (Figure 5.2A). Compared to males, females had less $25(OH)D_3$; $25(OH)D_3$ + $25(OH)D_2$ + $24,25(OH)_2D_3$; and calculated $24,25(OH)_2D_3$ + $25(OH)D_2$ in the synovial fluid (Figure 5.2B, 5.2C, 5.2D). However, using mass spectrometry, no differences in the levels of $24,25(OH)_2D_3$ between females and males were detected (Figure 5.2E).

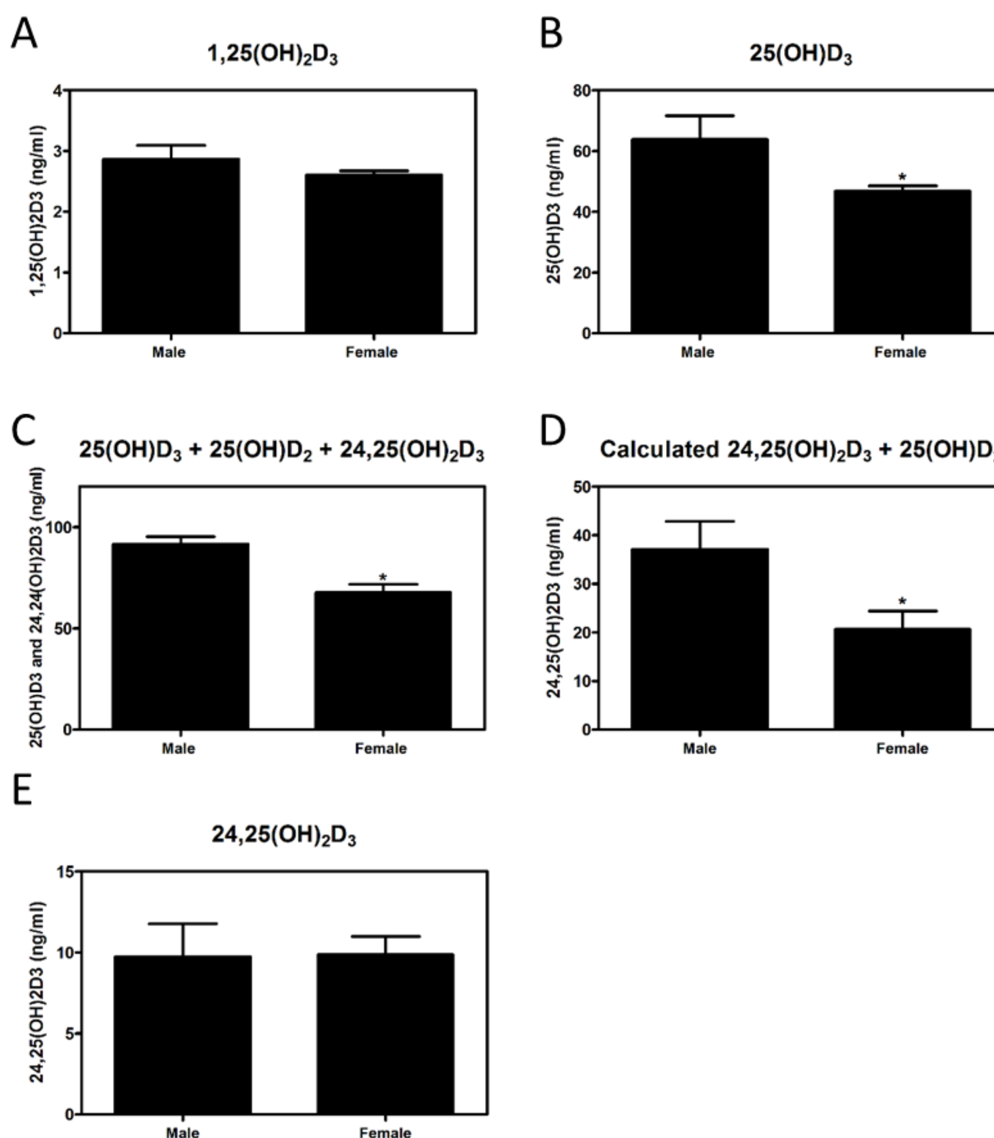

Figure 5.2: Synovial fluid levels of vitamin D metabolites were measured using ELISA (A-D) and mass spectrometry (E). *$p<0.05$ vs. male.

Chondrocytes

Chrondrocytes expressed mRNAs for both receptors for $1,25(OH)_2D_3$ (nVDR and PDIA3) and for both ERα66 (ESR1) and ERα36 (Figures 5.3A-D, respectively). Where fibrillation was minimal, female chondrocytes had less nVDR mRNA than male cells but comparable levels of mRNAs for the other three receptors. Female cells isolated from eroded cartilage had less nVDR mRNA, less PDIA3 mRNA, and more ESR1 mRNA than male cells.

mRNAs for inflammatory cytokines were elevated in male cells from eroded cartilage compared to cells from minimally fibrillated cartilage (Figures 5.3E-H). In contrast, female cells exhibited no differences in IL1a or IL1b mRNAs and expression of IL6 and IL8 was reduced in the female erosion cells compared to the minimally fibrillated cells. Female cells from minimally fibrillated cartilage had higher expression of IL1A, IL6 and IL8 than male cells from similar cartilage. No difference in expression of the pro-inflammatory cytokine IL10 was evident between male and female cells or as a function of the type of cartilage from which the cells were isolated (Figure 5.3I).

Sex-specific differences in expression of Wnt signaling molecules were observed, particularly in chondrocytes isolated from erosion cartilage. WNT3A, CTNNB, DKK2 and DKK2 were all expressed to a greater extent in male erosion cells than in cells from minimally eroded tissues (Figures 3J, L, M and N). This was also the case for CTNNB and DKK1 in cells from female erosion tissue compared to minimally fibrillated female cells (Figures 3L and M). However, WNT5A (Figure 3K) and DKK2 (Figure 3N) were reduced in female erosion tissue compared to minimally fibrillated tissue and the levels of these two mRNAs were significantly lower than seen in male cells from similar tissues.

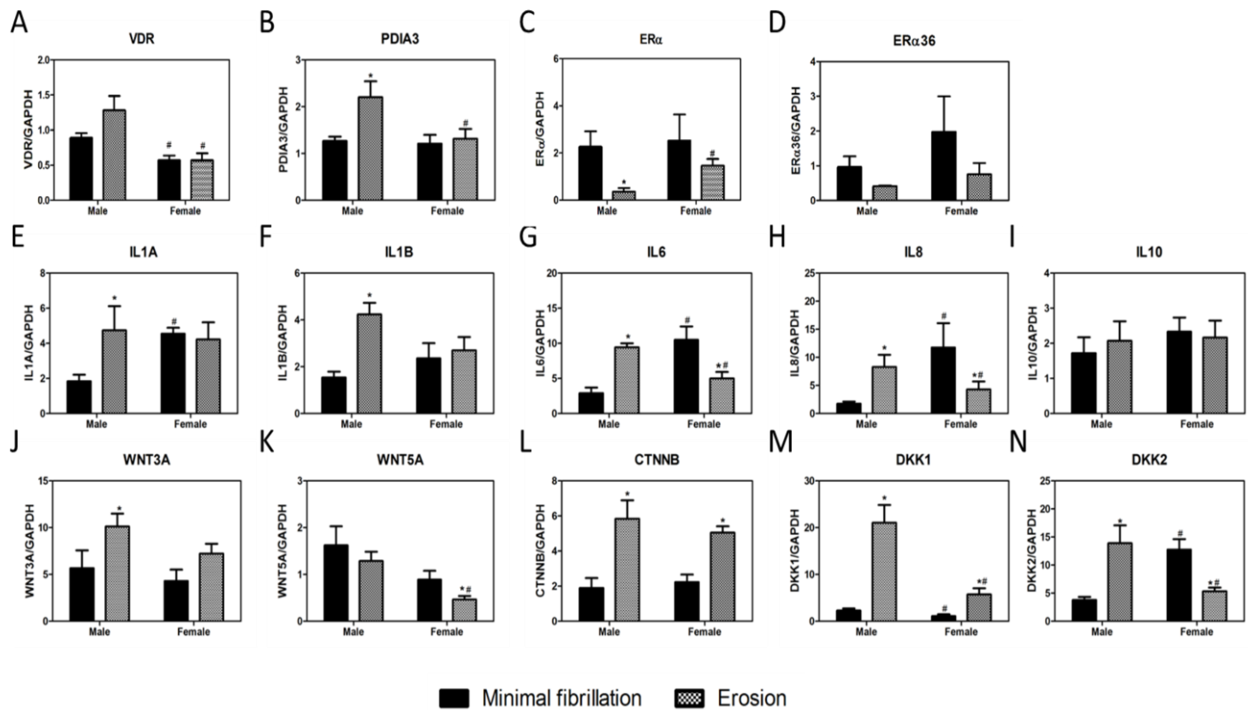

Figure 5.3: Phenotypic characteristics of female and male primary chondrocytes isolated from knee replacement tissues. mRNAs for $1\alpha,25(OH)_2D_3$ (VDR [a] and PDIA3 [b]) and estrogen receptors (ERα66 [c] and ERα36 [d]), interleukins (IL1A [e], IL1B [f], IL6 [g], IL8 [h] and IL10 [i]), and Wnt signaling molecules (WNT3A [j], WNT5A [k], beta-catenin [CTNNB] [l], DKK1 [m], and DKK2 [n]) were measured using real-time qPCR. *$p<0.05$ vs. minimal fibrillation; #$p<0.05$ vs. male.

Chondrocytes responded to stimulation by vitamin D metabolites in a sex-specific manner. Male cells exhibited a more robust increase in alkaline phosphatase activity in response to $1\alpha,25(OH)_2D_3$ than female cells (Figure 5.4A), but expression of ACAN was reduced (Figure 5.4B). No sex-specific differences in expression of COL2A1 or COMP were observed (Figures 5.4C, 5.4D). $24R,25(OH)_2D_3$ inhibited alkaline phosphatase activity in female chondrocyte cultures compared to control cells and compared to male cells (5.4E). $24R,25(OH)_2D_3$ had a stimulatory effect on male cells compared to control cultures and to female cells treated with the vitamin D metabolite (5.4F). $24R,25(OH)_2D_3$ affected male and female cells comparably with

respect to COL2A1 (increase) or COMP (decrease) (5.4G,H).

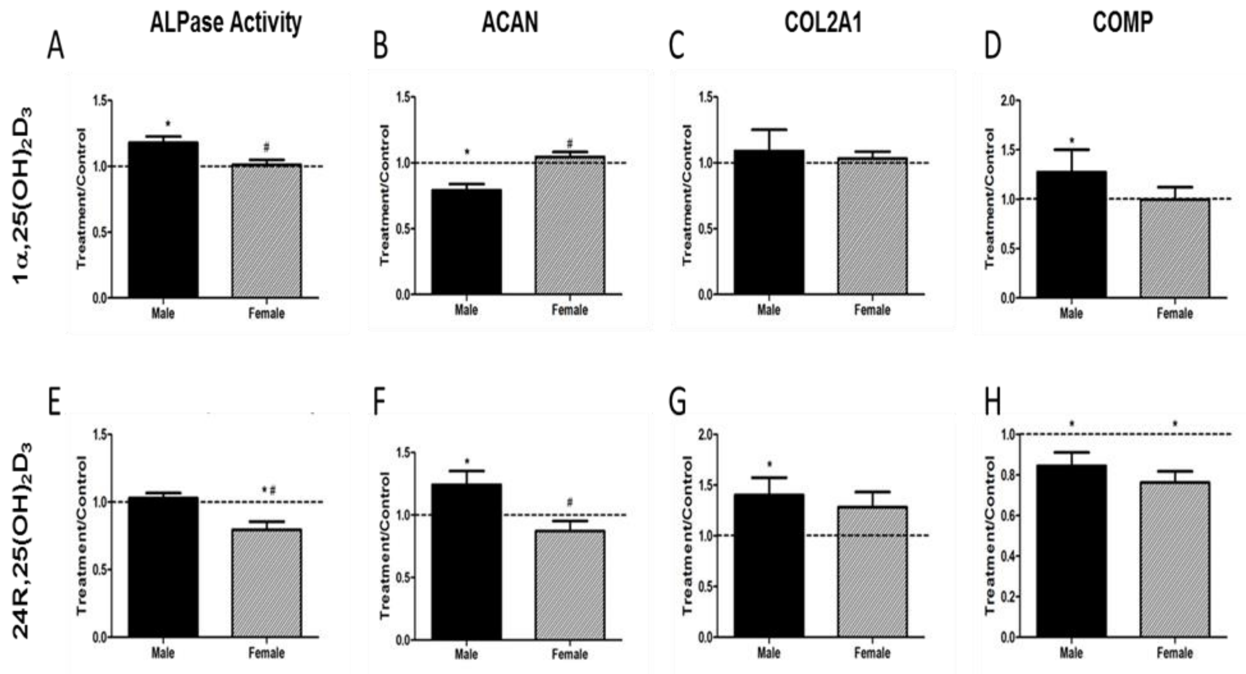

Figure 5.4: Response of passage 1 chondrocytes treated with 10^{-8} M 1α,25$(OH)_2D_3$ (A-D) and 10^{-7} M 24R,25$(OH)_2D_3$E2 (E-H). Alkaline phosphatase specific activity was measured in whole cell lysates. mRNAs for chondrocyte genes were measured using rea-time qPCR. Data show treatment vs. vehicle control ratios. The dashed line represents the vehicle control (dashed line=1). *$p<0.05$ vs. control; #$p<0.05$ vs. male.

E2 reduced alkaline phosphatase activity in male cells and stimulated it in female cells (Figure 5.5A). E2 had no effect on expression of ACAN, COL2A1 or COMP in male cells, but it increased expression of all three mRNAs in female cells (Figure 5.5C-D). DHT reduced alkaline phosphatase activity in male cells but had no effect on this enzyme in female cells (Figure 5.5E). DHT had no effect on expression of ACAN, COL2A1 or COMP in cells of either sex (5.5F-H).

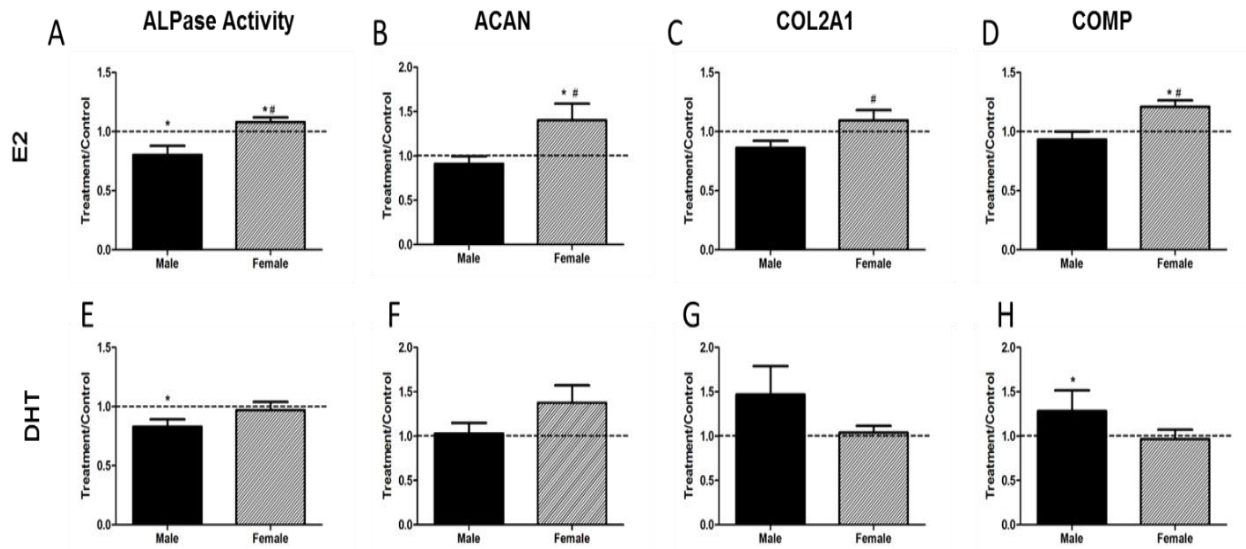

Figure 5.5: Response of passage 1 chondrocytes treated with 10^{-8} M E2 (A-D) and 10^{-7} M DHT (E-H). Alkaline phosphatase specific activity was measured in whole cell lysates. mRNAs for chondrocyte genes were measured using rea-time qPCR. Data show treatment vs. vehicle control ratios. The dashed line represents the vehicle control (dashed line=1). *$p<0.05$ vs. control; #$p<0.05$ vs. male.

Osteoblasts

Male osteoblasts from erosion tissue expressed high levels of nVDR compared to minimally fibrillated male cells or to female cells from either site (Figure 5.6A). No differences in expression of PDIA3 were observed (Figure 5.6B). Female cells expressed more ESR1 and more ERα36 than male cells (Figure 5.6C,D). However, no differences in expression of either E2 receptor were seen as a function of the cell source.

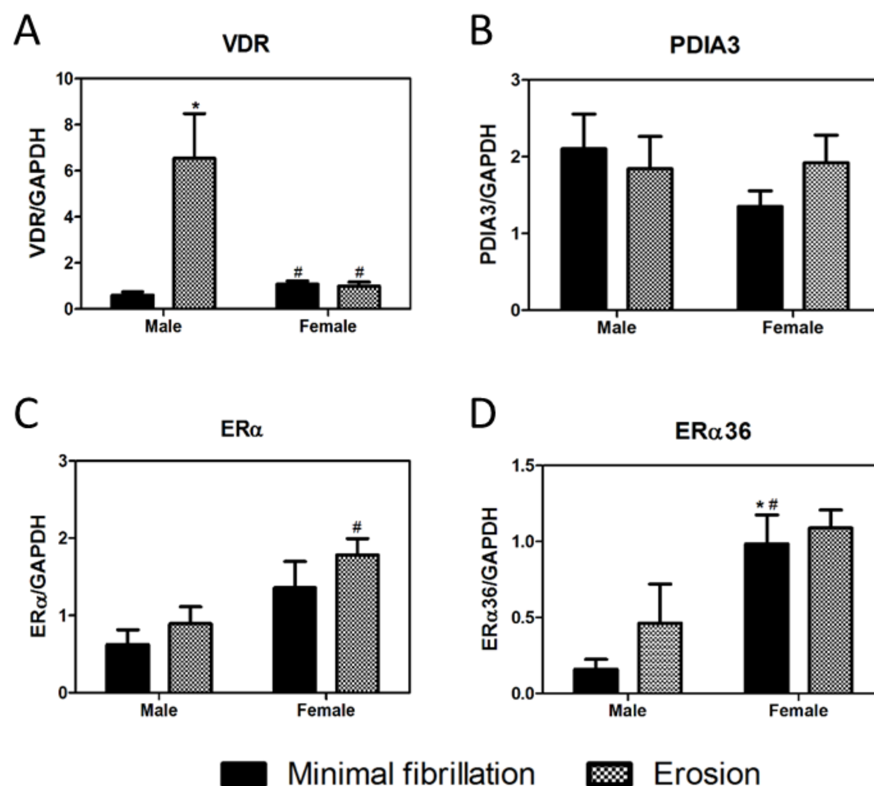

Figure 5.6: Female and male primary osteoblasts were isolated from subchondral bone. mRNAs for 1α,25$(OH)_2D_3$ (VDR (A) and PDIA3 (B)) and estrogen receptors ERa66 (ERa (C)) and ERa36 (D) were measured using real-time qPCR. *p<0.05 vs. minimal fibrillation; #p<0.05 vs. male.

In male osteoblasts, 1α,25$(OH)_2D_3$ increased alkaline phosphatase activity (Figure 5.7A) and osteocalcin production (Figure 5.7B), but decreased levels of osteoprotegerin (Figure 5.7C). 1α,25$(OH)_2D_3$ had no effect on alkaline phosphatase activity in female cells, but its stimulatory effect on osteocalcin and inhibitory effect on osteoprotegerin were comparable to that seen in male cells. E2 inhibited alkaline phosphatase activity in male cells (Figure 5.7D). It had no effect on enzyme activity in female osteoblast, but it stimulated osteocalcin production in the female cells (Figure 5.7E) and it inhibited osteoprotegern production (Figure 5.7F). In contrast, DHT increased alkaline phosphatase activity in male osteoblasts (Figure 5.7G), increased osteocalcin production (Figure 5.7H), and reduced osteoprotegerin production (Figure 5.7J). Female cells

exhibited increase osteoprotegerin production in response to DHT.

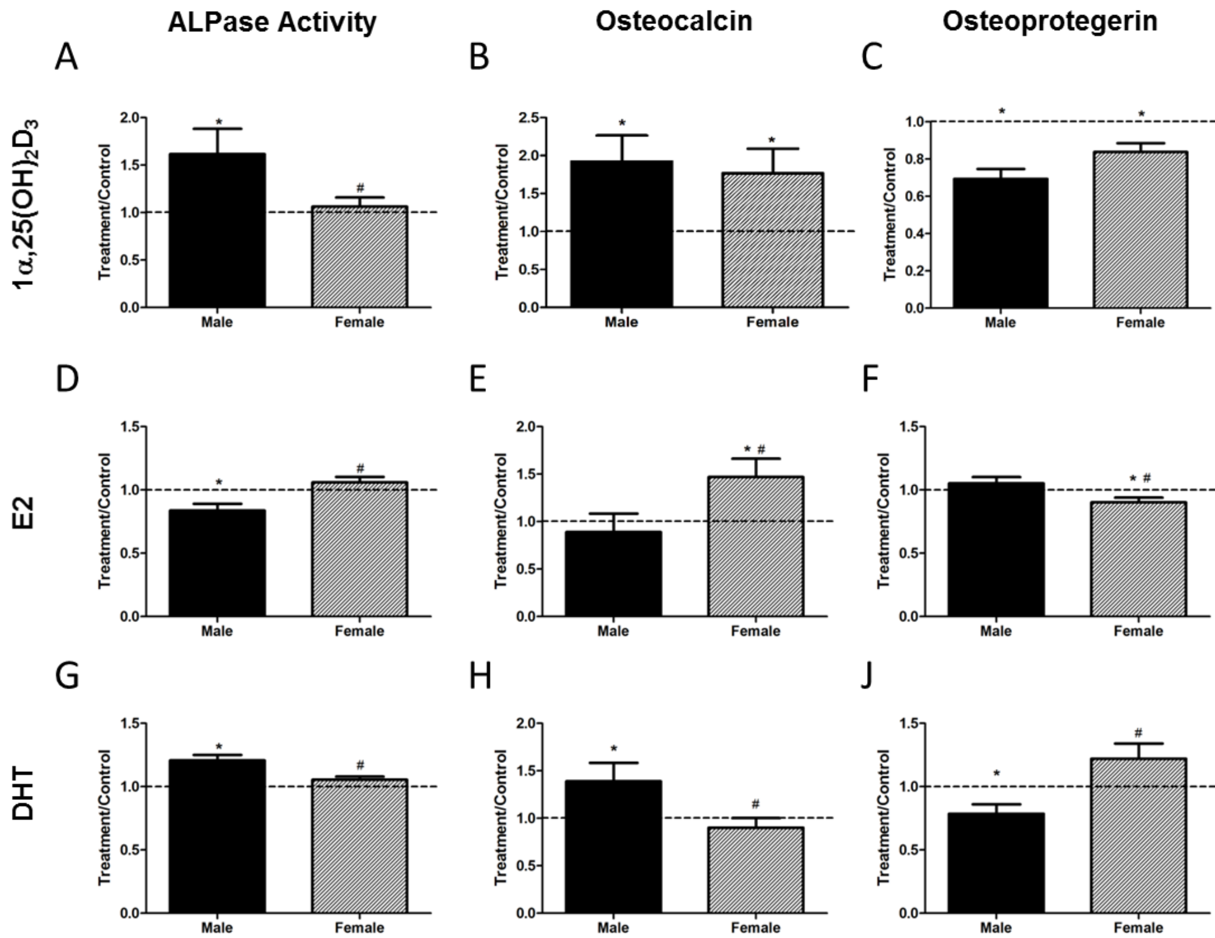

Figure 5.7: Response of passage 1 osteoblasts treated with 10^{-8} M $1\alpha,25(OH)_2D_3$ (A-C), 10^{-8} M E2 (D-F) and 10^{-7} M DHT (G-J). Alkaline phosphatase specific activity was measured in whole cell lysates. Osteocalcin and osteoprotegerin were measured using ELISA. Data show treatment vs. vehicle control ratios. The dashed line represents the vehicle control (dashed line=1). *p<0.05 vs. control; #p<0.05 vs. male.

DISCUSSION

This study used tissues and fluids obtained during total knee replacement surgery to investigate potential sex differences in knee tissues affected by OA. The severity of OA in the male and female patients was comparable. Thus, detected differences could be ascribed to

underlying sex-dependent traits, rather than differences in disease status. Our results demonstrate that sex differences are present in OA affected tissues. Not only are there differences in the composition of synovial fluid, but chondrocytes and osteoblasts isolated from OA affected cartilage and bone differ in a sex-dependent manner with respect to expression of hormone receptors and respond differentially to $1\alpha,25(OH)_2D_3$, $24R,25(OH)_2D_3$, E2 and DHT. Moreover, articular chondrocytes from male and female patients exhibit sex differences in their expression of genes for inflammatory cytokines, enzymes involved in matrix degradation, and Wnt signaling molecules involved in regulation of proliferation and differentiation.

The underlying etiology of OA is not well understood. It has been hypothesized that mechanical instabilities lead to changes in the biomechanical properties of the articular cartilage, and ultimately in the biochemical properties of the cells and extracellular matrix [103]. Recent data indicate that there are sex-specific differences at the molecular and cellular level that may exacerbate the effects of altered mechanical load [17, 30, 81]. Our patient population exhibited few clinical differences between men and women. There was no evidence of a difference in serum $25(OH)D_3$ levels, radiographic assessment, knee pain, or areas of knee pain prior to surgery between females and males. This could be due to the advanced stage of disease in the study population. Our power analysis determined that 10 males and 10 females would be sufficient to identify statistically significant differences, suggesting that sex-dependent clinical differences are relatively minor at this stage. Studies done on younger patients have shown sex differences in knees, with articular cartilage surface areas being 17.5% to 23.5% lower in women than in men [37]. This supports the idea that sex differences contribute to disease progression, but once advanced disease is established, sex differences are less evident.

We did observe some clinical differences in the study patient population that were sex-

dependent. The pain pressure threshold 1-cm above the medial joint line was significantly lower in females than males; the clinical significance of this is unclear. We did not test the pain pressure threshold of anatomic regions outside the medial knee and further study is warranted. We also found females were more likely to have used or are using vitamin D and estrogen supplements compared to males. Studies have linked estrogen supplementation with reduced risk of OA, which could explain how a drop in estrogen in postmenopausal women could contribute to severity of OA found in females [56, 133] compared to males. Together, these data suggest that future studies should include younger post-menopausal women and age-matched men to better assess sex differences in their disease severity.

Synovial fluid composition was affected by the genetic sex of the patient in a number of ways. Males had higher levels of matrix metalloproteinases, which are the primary enzymes responsible for the degradation of cartilage. They also had higher HGF (hepatocyte growth factor), which is synthesized by osteoblasts from the subchondral bone plate and produced at a higher rate by osteoarthritis osteoblasts [113]. However, males had higher levels of growth factors including TGF-βs, SCF (stem cell factor), and SCGF-beta (stem cell growth factor beta) and sGAGs compared to females, which have been shown to protect articular cartilage from degradation [57, 91, 151]. These results suggest that males may be more resistant to OA; although they had higher levels of matrix degradation enzymes, they also had more protective and growth inducing factors that could aid in the repair and remodeling processes. Females had higher levels of inflammatory cytokines, especially IL-18, which induces the production of PGE2 [44]. This is corroborated by studies showing that women experience more pain [130]. Females had less protective factors, which could explain their increased disease severity as they lose the protective effect of estrogen.

Factors that stimulate immune cells and macrophages were higher in synovial fluid from females than males. SCF and SCGF-beta have been shown to play a role in synovial mast cell proliferation and joint inflammation [24]. MIF is one of key regulators in acute and chronic immune-inflammatory conditions [75] and plays a crucial role in joint destruction and rheumatoid arthritis (RA) pathogenesis. Higher levels of TNF-β in female synovial fluids could be explained by the increase of macrophage factors found in females. During inflammation, macrophages can stimulate the production of TNF, a key cytokine that is thought to drive the inflammatory cascade in OA [73]. Females also had higher levels of TRAIL, which has been shown to induce chondrocyte apoptosis of chondrocytes and alters OA pathogenesis [84]. These results help elucidate why OA is more severe in females compared to males.

Vitamin D insufficiency is linked with weaker upper and lower limb strength and is associated with increased risks of falling in females [43, 96] and epidemiological studies have linked low levels of serum $25(OH)D_3$ with increased risk of knee OA [59, 93]. Although no sex differences in serum $25(OH)D_3$ were found, this vitamin D metabolite was reduced in synovial fluid from female patients compared to males. $25(OH)D_3$ is produced in the liver and is the precursor for subsequent metabolism of vitamin D in the kidney as well as in target tissues such as cartilage [104, 128]. Our finding that $25(OH)D_3$ was reduced in synovial fluid from females suggests that sex differences in vitamin D metabolite levels could be risk factors for OA [2, 163]. The ELISA kit used to measure $1,25(OH)_2D_3$, the active metabolite of $25(OH)D_3$ associated with Ca++ homeostasis as well as terminal differentiation of chondrocytes and osteoblasts [20, 21, 99], did not detect a difference between males and females. Because a suitable kit does not exist for measuring $24,25(OH)_2D_3$, an active metabolite associated with chondrocyte proliferation and resistance to apoptotic stimuli [18], we compared the results using two kits, one that measured

$25(OH)D_3$ alone and one that measured $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$. Both kits identified reduced vitamin D metabolites in female synovial fluid, suggesting a possible reduction in $24,25(OH)_2D_3$. However, when $24,25(OH)_2D_3$ was measured directly by a commercial lab using mass spectroscopy, no sex difference was found. None of these methods is definitive, but taken together they show a clear and reproducible finding that synovial vitamin D metabolites are regulated in a sex-dependent manner.

Cells isolated from both erosion cartilage and minimally fibrillated cartilage exhibited sex-specific phenotypic traits. In addition to differences in receptor expression discussed below, there were a number of differences with respect to expression of mRNAs for pro-inflammatory cytokines. The level of these cytokines was comparable for eroded cartilage regardless of sex; however, in minimally fibrillated cartilage females had higher levels of pro-inflammatory cytokines compared to males. This suggests that female cartilage may be produce higher levels of inflammatory cytokines at an earlier stage of osteoarthritis. Interestingly, the anti-inflammatory cytokine IL10 was expressed at comparable levels in cells from males and females irrespective of tissue source.

Expression of mRNAs for WNT family genes varied in a sex-specific manner as well, but the interpretation of these data will require further experimentation. Higher levels of Wnt antagonists are suggested to inhibit chondrocyte hypertrophy. DKK1 inhibits MMP-13 and ADAMTS-4 expression in chondrocytes in response to Wnt3a treatment [105]. Activation of Wnt/β-Catenin signaling in primary human chondrocytes, inhibits basal IL-1β stimulated increases in MMP-1, -3,-13 levels, possibly through inhibition of NFκB signaling [12, 85]. Males had higher levels of WNT signaling molecules, which is consistent with our findings that although males had higher levels of MMPs, they may be more resistant to OA as they also had

more factors that are protective.

Studies using growth plate chondrocytes and healthy human articular chondrocytes showed that the numbers of receptors vary in a sex-specific manner [39, 76, 94]. Our results examining gene expression also demonstrated sex-dependent differences. Chondrocytes from male erosion tissue and minimally fibrillated tissue expressed higher levels of mRNAs for the nuclear VDR whereas receptors for ERα66 (ESR1) were lower in cells from male erosion tissue than from female erosion tissue. Osteoblasts from male erosion tissue subchondral bone exhibited higher nuclear VDR expression and female osteoblasts had higher ESR1 than male cells. In addition, female osteoblasts from bone harvested from minimally fibrillated tissues had higher levels of the membrane-associated receptor for E2, ERα36. These findings could account for the differences observed in responsiveness to E2. They are supported by our previous observations using articular chondrocytes from normal males, which showed that male cells had no response to E2 whereas female chondrocytes exhibited a marked response to E2 [76].

We used passage 1 cells in the study to investigate the effect of hormone treatments on the cells. Human chondrocytes lose phenotypic traits when subpassaged, so it is preferable to use primary cells. Unfortunately, the number of primary cells was limited, necessitating the need for culture expansion. In a series of preliminary studies, we found that the first passage cells expressed similar levels of chondrocyte and osteoblast markers (data not shown). One of the limitations of our studies is that we could not get tissues from normal human knees as a control. However, we addressed the limitation by using cartilage and bone from areas that showed minimal fibrillation.

Increased levels of IL6 and IL8 and decreased levels of IL10 are linked with OA [4, 62, 72, 138]. In addition, IL1A, IL1B, IL6, and IL8 are up-regulated during inflammation that leads

to production of matrix metalloproteinases and matrix degradation [26]. In our study, males had higher levels of pro-inflammatory cytokines in erosion cells compared to the minimally fibrillated cells and compared to females, which could explain why males had higher MMPs found in their synovial fluids. IL-10 inhibits IL-1 and TNF-alpha expression [33]; thus its up-regulation in osteoarthritic chondrocytes may counteract the detrimental effects of these catabolic cytokines. Our results show that there were no differences in IL10 levels between males and females, and between erosion and minimally fibrillated cells, which could suggest that its protective effect on advanced osteoarthritic cells is lost.

Vitamin D metabolites play important roles in skeletal development [67] and in maintaining cartilage and bone homeostasis [35, 77]. Disruption in signaling by $1\alpha,25(OH)_2D_3$, either through the VDR or PDIA3, has been shown to result in altered cartilage and bone stability [16, 83]. Collectively, our data suggest that the difference in the levels of VDR and PDIA3 and local and circulating $25(OH)D_3$ between males and females could contribute to the more prevalent and severe cases of OA found in females.

Our results show that both male chondrocytes and osteoblasts were more responsive to $1\alpha,25(OH)_2D_3$ treatment than female cells, which could be due to greater number of receptors for $1\alpha,25(OH)_2D_3$ in male tissues. Previously, studies have shown that male osteoblasts have a more robust response to $1\alpha,25(OH)_2D_3$ [27], which supports our findings. Female cells were more responsive to estrogen treatment compared to males, which is supported by their greater expression of estrogen receptor mRNAs. The differences observed in cell responses to sex hormones could explain why females develop more severe OA than males. Studies have shown that chondrogenic progenitor cells respond differently to estrogen and testosterone in repairing OA cartilage, suggesting sexual dimorphism exists in cell repair mechanisms in OA [79].

Table 5.5. Primers for Real-Time PCR

Gene		Sequence
ACAN	F	TCA GCG GTT CCT TCT CCA G
	R	GCA GTT GTC TCC TCT TCT ACG
COL2A1	F	QuantiTect Primer Assay
	R	
COMP	F	CCT GCG TTC TTC TGC TCA C
	R	GCG TCA CAC TCC ATC ACC
CTNNB	F	GGC AGC AAC AGT CTT ACC
	R	TCC ACA TCC TCT TCC TCA
DKK1	F	CCA GAC CAT TGA CAA CTA CC
	R	CAG GCG AGA CAG ATT TGC
DKK2	F	TGA CTT GGG ATG GCA GAA TC
	R	CAG AAA TGA CGA GCA CAG C
ESR1	F	QuantiTect Primer Assay for ERα66
	R	
ERα36	F	GTGGTTTCCTCGTGTCTAAAGC
	R	GGTGTTGAGTGTTGGTTGCC
IL1A	F	TCC CGG GGC TTG CAC ACA CCT T
	R	ACT CTC CAC CCT GGC CCT GTT ACA
IL1B	F	TGG CAG AAA GGG AAC AGA AAG G
	R	AAC AAA AGG GCT GGG GAT TGG
IL6	F	CCT CGA GCC CAC CGG GAA CGA AA
	R	GGG GTA CTG GGG CAG GGA AGG C
IL7	F	TCT TCT TCT GTG CTG GAG ATG
	R	GGA CCT TGT TAT GCT GTT GC
IL8	F	GAC ATA CTC CCA AAC CTT TCC AC
	R	AAA CCT CTC CAC AAC CCT CTG
IL10	F	GGC TGA GGC TAC GGC GCT GTC A
	R	CTTCACCTGCTCCACGGCCTTGC
PDIA3	F	AAGAAGAAGGCACAGGAG
	R	ATGAACTTCAGGGTCAGC
VDR	F	CAT CAG AAG GAG AAG GAA GG
	R	TGA GGC AAC AGC ATT ATC C
WNT3A	F	CTGTAGCGAGGACATCGAGTTT
	R	GGCACCTTGAAGTAGGTGTAG
WNT5A	F	TCT CAG CCC AAG CAA CAA GG
	R	GCC AGC ATC ACA TCA CAA CAC

CONCLUSION

In this study, we identified sex-specific differences synovial fluid, chondrocytes and osteoblasts in advanced human knee osteoarthritis tissues. Our understanding of such sex-

differences is still very early and additional research is needed. Understanding such sex differences in knee osteoarthritis may allow for the introduction of sex-specific therapies and new treatment avenues for patients suffering from this disabling condition.

CHAPTER 6

CONCLUSION AND FUTURE PERSPECTIVES

The major hurdles in osteoarthritis research include elucidating the mechanisms of disease, determining methods for early detection, and developing strategies for intervention and disease modification. In our study, we examined the effects of 24R,25$(OH)_2D_3$ in both *in vitro* and *in vivo* models of OA. Our results showed that 24R,25$(OH)_2D_3$ prevented cartilage degeneration during disease progression and mediated local inflammatory condition. 24R,25$(OH)_2D_3$ was shown to inhibit chondrocyte apoptosis, reduce production and activity of catabolic factors, and modulate inflammatory responses that are contribute to carriage regeneration. The effects of 24R,25$(OH)_2D_3$ is similar to that of TGF-β1. Although 24R,25$(OH)_2D_3$ was shown to enhance TGF-β1 signaling, the potential mechanisms of and the potential mechanism of 24R,25$(OH)_2D_3$'s action needs further elucidation (Figure 6.1).

24R,25$(OH)_2D_3$ reduced cartilage degeneration in our animal model at the end of four weeks. Results from this study provide insights into how a potential therapy could be developed using 24R,25$(OH)_2D_3$ injections. Our results show that the intraarticular delivery of 24R,25$(OH)_2D_3$ also showed systemic effects in reducing pro-inflammatory factors and increasing anti-inflammatory factors. Furthermore, 24R,25$(OH)_2D_3$ was show to mediate levels of several growth factors including TGF-β1 and VEGF. Future studies need to further examine the role of 24R,25$(OH)_2D_3$ in mediating the immune response and its interactions with other major factors involved in OA pathogenesis.

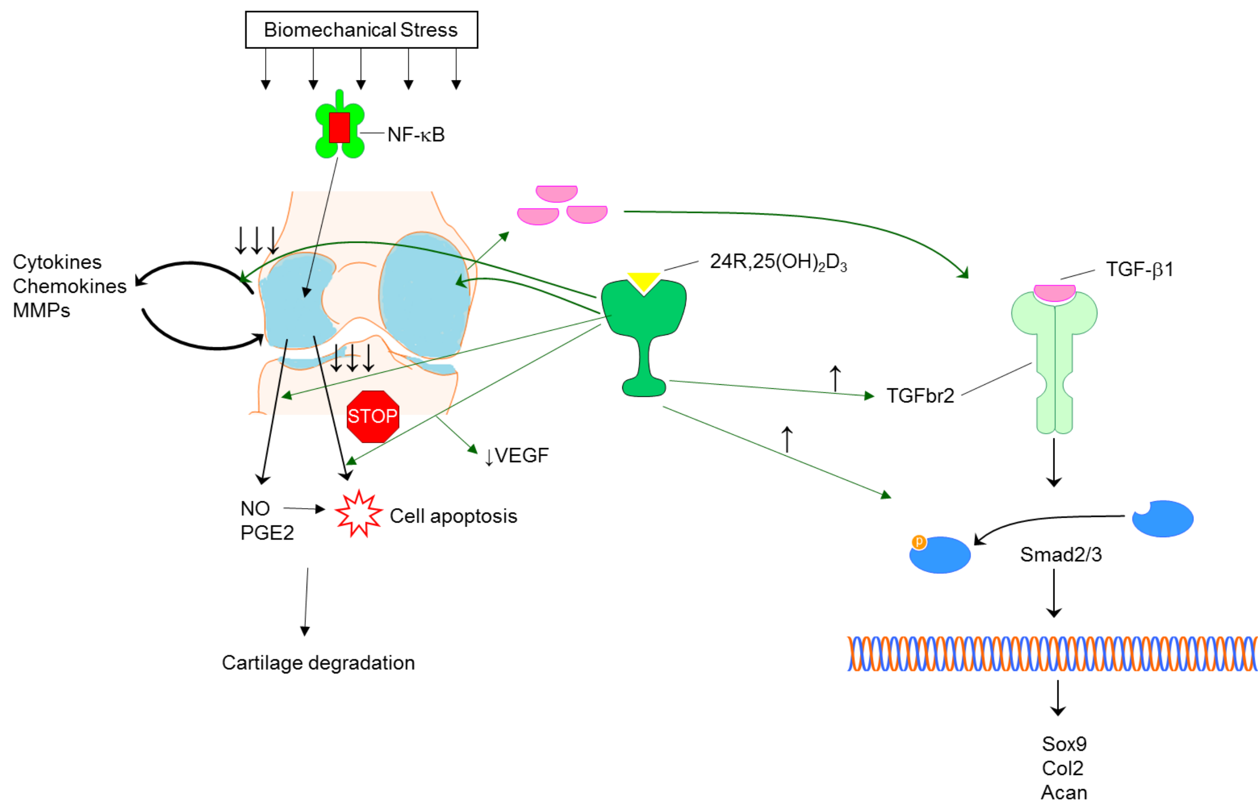

Figure 6.1 Schematic of 24R,25(OH)2D3 and TGF-β1 signaling pathways in mediating cartilage remolding.

Results from our human OA Our study demonstrate potential sex differences in the levels of vitamin D_3 metabolites and OA tissues response to vitamin D_3 metabolites treatment. These results provide explanations to the sex differences in OA prevalence and severity observed in patients. In order to tailor future OA therapies using vitamin D_3 to be patient and sex specific, future studies elucidating the relationship between growth hormones and vitamin D_3 interactions during OA disease progression are needed.

Study of osteoarthritis incidence and prevalence shows that the most important risk factors are age, excessive joint loading, and injury. Our studies examined three different osteoarthritic conditions. The cell model with IL-1β mimics how initially inflammation can

affect chondrocyte metabolism and its response to growth factor and hormone. Results from this study help us understand how $24R,25(OH)_2D_3$ mediates chondrocyte response to stimulation and the mechanism of its action. The *in vivo* model simulates post-traumatic injury OA development. Results from this study demonstrate onset OA due to imbalance of mechanical forces and joint injury that are commonly seen in young OA patients. These patients will respond differently to OA treatments compared to elder patients whose hormone and hormone receptor levels have altered and may be less sensitive to $24R,25(OH)_2D_3$ treatment. Sex specific factors were observed in post-menopausal patients that underwent joint replacement surgery and may provide insights into how hormones may affect the incidence of disease with age. Cells isolated from more advanced OA tissue regions showed less sensitivity to $24R,25(OH)_2D_3$ treatment. Collectively, results from this study provide answers to better elucidate lead to effective methods of preventing osteoarthritis and slowing its progression.

The major unmet medical challenges in intraarticular drug delivery are lack of therapeutic drugs and short duration of drug effectiveness due to rapid uptake by the circulation and turnover of synovial fluid. Tissue engineering and drug delivery systems are the fields that deal with these challenges and provide an alternative method for treating OA. Future studies need to consider engineer a drug delivery system that can couple the therapeutic effects of $24R,25(OH)_2D_3$ and a carrier system to relieve joint cartilage degeneration and provide an accurate and sustained long-term release of therapeutically desirable drug levels.

Two of the major functional specifications for the drug delivery system will be its stimuli-responsive release mechanism to for local sustained delivery and its biocompatibility. The therapeutic efficacy depends on the retention time of the drug in the joint. One of the challenges with intraarticular drug delivery is the presence of lymphatic system that rapidly

eliminates particles from the synovial cavity [86]. Studies have shown that drugs administrated as solutions or suspension have half-lives that can vary between 1 and 2 hours, thus requiring frequent administration of the therapeutic drug (weekly for 3-6 weeks). Frequent injections can lead side effects and patient discomfort. To increase the drug residence time in the synovial space, drug delivery strategies such as liposome carriers, cross-linking and microencapsulation nanoparticles have been explored. Modifications to increase molecular weight such PEGylation have demonstrated to enhance treatment of knee osteoarthritis [3].

One of the possible ways to increase drug retention is to use polyketal nanoparticles (TKNs) to load the therapeutic drug. This system has been shown to have a lower cytotoxicity profile than the FDA-approved material PLGA in treating inflammation. Sites of inflammation, such as joints affected by OA, have abnormally high levels of reactive oxygen species (ROS). TKNs delivered to the inflamed joints will be cleaved at the ROS sensitive side chains, thus triggering release of the encapsulated [159] (Figure 6.2). The cationic lipid based encapsulation formula endows the nanoparticle with a positive surface charge and can enhance drug uptake through adhesion to the negatively charged articular cartilage surface [140]. The biostability of the TKNs and its electrostatic adhesion to cartilage enable sustained release of encapsulated drugs, which solves the shortcoming of current drug delivery methods in treating osteoarthritis. Encapsulation of vitamin D within the TKNs using a cationic lipid double emulsion technique with the above criteria. The release profile of the nanoparticles can be studied by incubating them in superoxide solutions and cell culture media as controls. The controlled release can be monitored using gel permeation chromatography to measure its molecular weight. The above designed nanoparticle-based delivery scheme for sustained delivery of vitamin D will help increase retention time in the joint cavity and enhance specific

release upon inflammatory stimuli. In our study, we delivered physiological relevant concentrations of 24R,25$(OH)_2D_3$ weekly for four weeks. Future studies with the TKN carrier system, can load a higher centration of 24R,25$(OH)_2D_3$ for a one time delivery treatment.

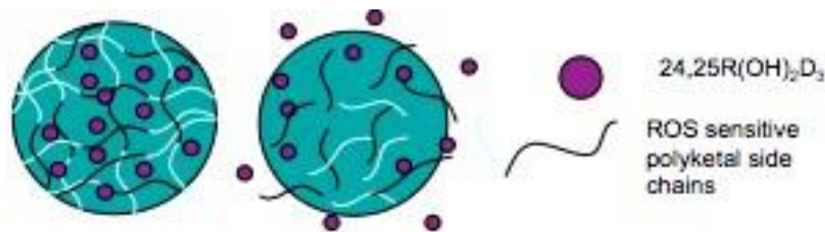

Figure 6.2 Schematic TKNs with encapsulated vitamin D. ROS cleaves the polyketal linkages releasing drug.

Developing a novel drug delivery therapy that stimulates cartilage regeneration and can be tailored to be patient specific may take decades before it is clinically available to any patient. However, evaluating and tailoring intraarticular therapies for tissue regeneration via studying 24R,25$(OH)_2D_3$ in both animal models and clinical may lead to the next clinical breakthrough for regenerative medicine.

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