“We learn more by looking for the answer to a question and not finding it than we do from learning the answer itself.” - Lloyd Alexander

University of Alberta

Collagen I: an aberrantly expressed molecule in chondrocytes or a key player in tissue stabilization and repair both in vivo and in vitro?

by

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DEDICATION

I would like to dedicate this work to Zig Stogowski for starting me on the path to higher education but also for seeing something inside of me that I couldn't see myself.

ABSTRACT

Extrinsic repair techniques for the treatment of acute chondral injuries continue to yield suboptimal repair. The inability of these techniques to produce hyaline cartilage underscores the limitations in our understanding of basic chondrocyte biology. Conversely, intrinsic repair tissue has not been extensively studied despite the fact that it can yield hyaline-like cartilage and is commonly observed in osteoarthritis. Attempts at extrinsic repair could therefore benefit from a better understanding of the successes and failures inherent in the intrinsic repair process.

Chondrocyte culture has typically been conducted under non-physiologic conditions whereby chondrocytes readily dedifferentiate. Consequently, much of the knowledge gained about chondrocytes has been misleading thus hindering advancements in chondrocyte biology and attempts at extrinsic articular cartilage (AC) repair. Hypoxic culture conditions, which are beneficial towards the preservation of the chondrocyte phenotype, remain insufficient due to elevated collagen I gene expression. As such, an appropriate model system does not yet exist in which to study physiologically-relevant chondrocyte biology.

The presence and prevalence of collagen I in both degenerate and *de novo* osteoartritic tissue was examined immunohistochemically. Collagen I deposition during osteoarthritic progression was compared against IHC staining for collagen II and aggrecan. A novel model system was also evaluated for chondrocytic phenotype retention. To this end, hypoxic, high-density-monolayer-chondrocyte

(HDMC) cultures were compared to freshly isolated chondrocytes for their ability to maintain a chondrocytic extracellular matrix (ECM) gene expression profile.

HDMC culture conditions prevented the severe loss of the phenotype typically associated with conventional monolayer culture. Moreover, prolonged HDMC culture resulted in the formation of a complex ECM and a marked suppression of collagen I expression. This study also demonstrated that collagen I deposition occurs in osteoarthritic AC at the onset of structural damage and increases in response to increasing structural damage. Collagen I deposition was also found in different types of *de novo* cartilage associated with osteoarthritic joints and suggests that it plays an important role in intrinsic cartilage repair. Taken together, this work demonstrates that collagen I is a common feature in the ECM of structurally immature and structurally damaged AC and hence may play a role in tissue stabilization.

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LIST OF ABREVIATIONS

2D	Two dimensional
3D	Three dimensional
AC	Articular cartilage
ACI	Autologous chondrocyte implantation
ACL	Anterior cruciate ligament
ADAMTS	A disintegrin and metalloproteinase with a thrombospondin type 1 motif
AEC	Alginate embedded chondrocytes
ANOVA	Analysis of variance
A^o	Angstom
BMP	Bone morphogenic protein
C	Carboxy
Cbfa1	CCAAT-binding factor alpha 1 transcription factor
CCD	Charge-coupled device
CDM	Chondrocyte differentiation medium
cDNA	Complementary dexoriobonucleic acid
CGM	Chondrocyte growth medium
COMP	Cartilage oligomeric protein
CS	Chondroitin sulfate
Da	Dalton
DAB	3,3'-Diaminobenzidine
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
F12	Ham's F12 nutrient medium
FACIT	Fibril-associated collagens with interrupted triple helicies
FAK	Focal adhesion kinase

FIC	Freshly isolated chondrocytes
g	Relative centrifugal force
G1	Globular domain one
G2	Globular domain two
G3	Globular domain three
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gly	Glycine
H&E	Hematoxylin and eosin
H_2O_2	Hydrogen peroxide
HDMC	High density monolayer chondrocytes
HHGS	Histological/Histochemical Grading System
HIF	Hypoxia-inducible factor
HOPE	Human Organ and Procurement Exchange
HRE	Hypoxia responsive element
HSP	Heat shock protein
IGD	Interglobular domain
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IIA	Adult splice variant of collagen II alpha one chain
IIB	Embryonic splice variant of collagen II alpha one chain
IL-1	Interleukin one
JNK	c-jun NH_2-terminal kinase
KS	Keratan sulfate
MACI	Matrix-induced chondrocyte implantation
MAPK	Mitogen-activated protein kinase
MG	Mankin grade
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
N	Amino
O2	Molecular oxygen

OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OB	Outerbridge
P0	Passage zero
PBS	Phosphate buffered saline
pH	Potential hydrogen
PKC	Protein kinase C
PLGA	poly(lactic-*co*-glycolic acid)
PTR	Proteoglycan tandem repeats
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SAPK	Stress-activated protein kinase
SFA	Societe Francaise d'Arthroscopie
SLRP	Short leucine-rich proteoglycan
SMCD	Schmid type metaphyseal chondroplasia
SOX	SRY box transcription factor
SPSS	Statistical package for the social sciences
TGF	Transforming growth factor
TIMP	Tissue inhibitors of MMPs
TKA	Total knee arthroplasty
TNF	Tumor necrosis factor
α-1	Alpha 1 chain of a collagen molecule
α-2	Alpha 2 chain of a collagen molecule
α-3	Alpha 3 chain of a collagen molecule

CHAPTER ONE: Introduction

1.1 Project Rationale

The emergence of autologous chondrocyte implantation (ACI) in the mid-1990s provided orthopedic surgeons with the first cell-based, tissue engineering approach for the treatment acute chondral injuries. Based on initial experimentation, this exciting new technique was expected to generate high quality, *de novo,* hyaline cartilage and therefore was met with considerable optimism(1). Unfortunately; however, for reasons that remain unclear, much of the tissue resulting from the clinical application of this promising technique was of inferior quality and closely resembled fibrocartilage(2,3). The discrepancy between the observed and expected results for this technique underscores the limitations that exist in the collective understanding of chondrocyte biology at present.

To better understand chondrocyte biology in general, as well as to gain insight into the factors responsible for the development of the inferior quality ACI repair cartilage, various subject areas have been explored including: scaffold engineering(4-7), cytokines stimulation(8-10), mechanotransduction(11,12), cell density(13,14) and even hypoxia(15-17). While a combination of these potential variables may ultimately be responsible for inducing the ACI-related production of fibrocartilage, the observation that chondrocytes in 2D culture readily dedifferentiated into fibroblast-like cells(18-20) offers one compelling possible mechanism by which these factors may operate. Because ACI relies on an *ex vivo* chondrocyte propagation step, it is conceivable that during this step the chondrocytes dedifferentiate into collagen I-producing fibroblast-like cells and yield cartilage with a composition closely resembling collagen I-rich fibrocartilage.

Interestingly, the fibrocartilagenous tissue that is produced during attempts at extrinsic repair such as ACI(21), appear to be similar to a tissue that is commonly found in the joints of individuals suffering with advanced OA(22). It is likely that

a better understanding of this tissue and its evolution during the course of OA will yield novel insight into the limitations that exist clinically for the generation of *de novo* articular cartilage. Unfortunately, the structure and molecular composition of OA-related fibrocartilage has not been extensively studied making direct comparisons impossible.

The presence of *de novo* fibrocartilage in OA joints, which are typically characterized by destructive catabolic processes(23), yields somewhat of a homeostatic paradox and dictates that within OA joints there should be clear molecular evidence of anabolic processes. Synthesis of any new cartilage would require the expression, export and successful integration of a variety of ECM components. While much has yet to be demonstrated, studies have shown that collagen II and aggrecan gene expression are frequently upregulated in the late stages of OA(24-26). It is possible that such alterations in gene regulation, within heavily eroded AC, may represent abnormal processes or the activation of a salvage pathway; however, if present in early stages of OA, increases in ECM expression would provide evidence that anabolism and perhaps even intrinsic repair are inherent properties of AC.

1.2 Objectives

A detailed understanding of the factors that lead to the production intrinsic repair tissue in OA, coupled with knowledge of the factors responsible for maintaining chondrocyte differentiation in culture should translate into improved clinical outcomes with ACI. As a result, the objectives for this body of work are as follows:

1. examine IHC disease progression in early to intermediate OA for evidence of anabolism,
2. examine the prevalence, quality and composition of *de novo* repair tissue in early to intermediate OA,
3. establish the presence of collagen I in early to intermediate OA and determine its distribution throughout the AC,

4. assess the qualitative differences between histological and IHC staining in OA to determine the necessity of an IHC scoring system,
5. test several promising combinations of niche factors on the ECM gene expression levels in cultured chondrocytes to determine which is superior for the maintenance of the chondrocytic phenotype.

1.3 Literature Review

1.3.1 Connective Tissue

Connective tissue is the most abundant and widely distributed tissue in the body. It is very heterogeneous category of tissue type and, as such, it serves a broad array of functions throughout the body [reviewed in (27)]. Connective tissue can be subdivided into 3 tissue types 1) connective tissue proper 2) fluid connective tissue 3) supporting connective tissue. All connective tissues arise from embryonic mesenchyme and are composed of 3 basic components: 1) specialized cells 2) extracellular protein fibers 3) ground substance, a complex fluid-like component that surrounds and suspends the cells and fibers. Despite having three components, connective tissue is usually described as having a cellular component and an extracellular component (matrix) comprised of protein fibers and ground substance. Both the cells and their associated matrix vary greatly from one tissue subcategory to another and give rise to loose and dense connective tissue, blood, lymph, bone and cartilage(27).

1.3.2 Cartilage

Cartilage, which is very abundant in the developing embryo is greatly restricted in distribution in an adult; however, it can be found in a variety of locations throughout the body. There are 3 types of cartilage: 1) fibrocartilage 2) elastic cartilage and 3) hyaline cartilage. Fibrocartilage is unique in its ability to resist both strong compression and strong tension forces and as a result it can be found in such locations as the intervertebral disks of the spine, meniscus of the knee and pubic symphysis of the pelvis [reviewed in (28)]. Although not as strong, elastic cartilage is incredibly resilient due to a high density of special elastic fibers and

can easily tolerate repeated bending. This type of cartilage can be found in such locations as the ears, nose and epiglottis. Hyaline cartilage is the most abundant form of cartilage. However, it is also arguably the weakest of the three. Although hyaline cartilage is present in the nose, is more commonly associated with the ribcage and synovial joints. While most cartilage is surrounded by a layer of dense irregular connective tissue known as a perichondrium, the special type of cartilage in synovial joints, known as articular cartilage (AC) is devoid of this supportive and regenerative layer(28). As a result, the AC found in a synovial joint, in contrast to the various types of cartilage found in the rest of the body, is unable to repair itself effectively(29).

Unlike most tissues, cartilage is devoid of nerves as well as blood and lymphatic vessels. Given the avascularity of this tissue, cartilage must depend on diffusion for its mineral and nutrient supply. Recently, several antiangiogenesis factors (ie. Endostatin, Chondromodulin-I) have been discovered in adult cartilage that are believed to prevent the invasion of blood vessels thus maintaining the avascular environment(30,31). Furthermore, since both immunoglobulins and monocytes are sterically excluded from entering the cartilage due to the density of the extracellular matrix (ECM), it is generally considered to be an immune-privileged site(29).

The cells that reside within and maintain the matrix components of adult cartilage are known as chondrocytes. These cells exist within tiny cavities in the matrix known as lacunae and are fully differentiated with limited capacity to replicate(29). Unlike chondrocytes however, the chondroblasts from which they are derived are the cartilage precursors in the developing fetus and retain the potential to replicate. These chondroblasts are believed to originate from the mesenchyme which arises early in embryologic development.

1.3.3 Synovial Joints

Synovial joints are the most movable class of joints in the body. They permit locomotion and endow the limbs with a broad range of movement. In addition to the AC which covers the ends of the long bones, synovial joints are comprised of an array of other structures. Most of these associated structures are present to sustain and protect the integrity of the articular cartilage, without which the joint could not function properly.

A typical synovial joint is contained within a tough articular capsule. The articular capsule is comprised of a thick outer layer known as the fibrous capsule and a thin inner layer known as the synovial membrane. The fibrous capsule provides strength and support for the synovial membrane which is responsible for producing the synovial fluid. The synovial fluid which is located throughout the joint cavity serves two main functions; it lubricates the joint and provides all of the necessary nourishment to sustain the chondrocytes. Surprisingly, the typical adult knee contains less than 4 ml of synovial fluid which is barely enough to cover the internal joint surfaces with a layer of fluid 20 μm deep(29).

Although tough, the articular capsule itself is not adequate to maintain proper joint alignment and hence function. As a result, synovial joints also have a series of strong ligaments that surround the articular capsule and are designed to hold the respective bones in the proper configuration. Failure to maintain proper alignment has been shown to lead to abnormal cartilage wear**.**

1.3.4 The Knee Joint

The knee joint is a special type of synovial joint because, due to its shape and function, it has additional specialized structures that are associated with it. In addition to the general structures associated with all synovial joints, the knee joint also possesses intracapsular anterior and posterior cruciate ligaments, C-shaped medial and lateral menisci and a small partially cartilage-covered bone, known as the patella, which lies immediately anterior to the joint. In addition to the extracapsular ligaments, the cruciate ligaments are instrumental in maintaining

proper joint alignment because they function like x-shaped restraining straps between the femur and tibia. Studies in canines have shown that simple disruption of one or both of the cruciate ligaments can lead to a dramatic increase in cartilage wear and even signs of premature arthritis(32,33)**.** The two menisci, which are composed of fibrocartilage, provide additional stability and cushioning to the knee, but perhaps more importantly serve as a guide to the femur and tibia as they slide past one another during flexion and extension.

The patella, which is held in place by the patellar ligament and quadriceps tendon, articulates with two distinct regions of cartilage on the distal anterior portion of the femur which are known as the femoral condyles. Although much of the distal end of the femur is covered by articular cartilage, the femoral condyles are the functional regions of that cartilage. Since any damage to these functional portions of the articular cartilage can result in cartilage deterioration and loss of function, it is fitting that these regions are provided the extra protection by the patella.

1.3.5 Articular Cartilage

The primary function of articular cartilage is to provide a smooth surface for the articulation of the bones against one another. It is able to achieve this because it has one of the lowest coefficients of friction known for any surface to surface contact (Table 1.1)**.** As a result, no synthetic material has been discovered to date that performs as well as a joint surface. The remarkable ability of this unique tissue ultimately arises from its structure and composition.

1.3.5.1 Constituents

Like the body as a whole, cartilage is comprised of approximately 70-80 % water but that is one of the few commonalities that cartilage has with many of the other tissues in the body (Fig. 1.1).

Table 1.1. Coefficient of friction for different materials [adapted from Dowson(34)].

Material	Coefficient
Gold on gold	2.8
Aluminum on aluminum	1.9
Silver on silver	1.5
Glass on glass	0.9
Steel on steel	0.7
Brass on steel	0.35
Wood on wood	0.25
Nylon on nylon	0.2
Graphite on steel	0.2
Ice on ice at 0^{o}C	0.1
Cobalt chrome on polyethylene	0.01 to 0.05
Normal Cartilage	0.005 to 0.02

While many tissues like the liver, heart and kidneys are primarily comprised of cells that perform the tissue's primary function, cartilage is composed almost entirely of extracellular matrix. Perhaps not surprisingly in the case of cartilage, it is the ECM, and not the cells themselves, that performs the primary frictionless load-bearing function of the tissue. In fact, chondrocytes are in the extreme minority in articular cartilage and typically comprise less than 5% of the tissue(35).

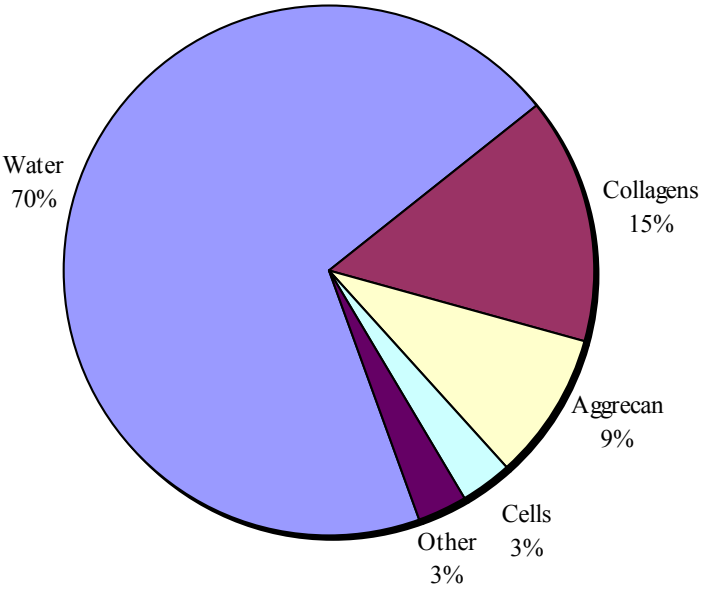

Figure 1.1. Molecular composition of articular cartilage (Adapted from Aigner and Stove, 2003)(35).

The cells that reside in this sparsely populated matrix nonetheless play a very important role in maintaining it. Chondrocytes are responsible for excreting, maintaining and where necessary, remodeling the massive amount of extracellular matrix that encases them. It is however not simply a matter of continually extruding copious amounts of the components that make up the ECM, but rather

producing just the right amounts at the correct times. If the chondrocytes fail to get this complex recipe right, then the tissue will not possess the appropriate stiffness and resilience to function properly and the ECM will begin to breakdown. Although the ECM is primarily comprised of collagen II and aggrecan, there is a long list of minor constituents (Table 1.2)

Table 1.2. Constituents that comprise articular cartilage

Collagens	Proteoglycans	GAGs	Proteins
Col II	Aggrecan	Chondroitin Sulfate	Link Protein
Col III	Biglycan	Keratan Sulfate	Hyaluronate
Col VI	Decorin		Fibronectin
Col IX	Fibromodulin		COMP
Col X*	Lumican		
Col XI			
Col XII			
Col XIV			
Col XVI*			

(adapted from Aigner and Stove, 2003 and Eyre, 2004) * taken from Gelse 2003

Many researchers tend to focus on the major cartilage constituents. However; the minor constituents, although more difficult to detect, are likely to play an equal or perhaps even more important roles in determining overall cartilage structure and integrity than their more abundant counterparts. The smaller non-aggregating proteoglycans such as biglycan, decorin and fibromodulin, for example, are believed to be involved in cross linking the various component of the ECM(35). Without adequate crosslinking, the physical properties of the tissue will be adversely impacted and it will ultimately fail to withstand the day to day stresses placed upon it.

1.3.5.2 Major Cartilage Zones

Articular cartilage is composed of a cellular fraction consisting of chondrocytes and an extracellular matrix. Although the exact composition of the various constituents differs between the different types of cartilage it also differs with depth in articular cartilage. As a result, there are 4 distinct layers that can be identified: 1) superficial/tangential layer 2) transitional layer 3) deep/radial layer 4) layer of calcified cartilage (Fig. 1.2). As the name implies, the superficial layer is the uppermost zone and is characterized by: chondrocytes which are elongated in a direction parallel to the articular surface, thin collagen fibrils arranged parallel to the cartilage surface, a low density of proteoglycans and high water content. The transitional layer is the largest zone and is characterized by larger diameter collagen fibrils, which surround less elongated chondrocytes. In sharp contrast the superficial layer, the deep layer contains large collagen fibrils oriented perpendicular to the surface, rounded chondrocytes, the highest density of proteoglycans and the lowest water content.

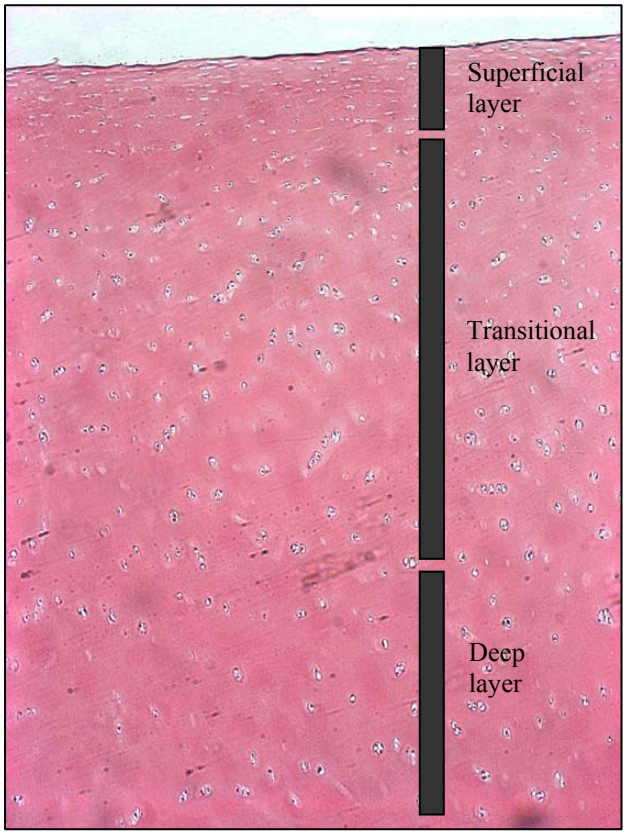

Figure 1.2. Hematoxylin and eosin stained section of normal articular cartilage illustrating the three major layers.

The deepest layer of cartilage which rests on the subchondral bone is known as the layer of calcification and is characterized by smaller cells in a cartilaginous matrix which have been encrusted by apatitic salts. Separating the deep layer from the layer of calcification below it is a line parallel to the surface known as the tide

mark. According to Smith(36) the chondrocytes are non-randomly distributed within the matrix and their metabolic profile is determined by their depth within the cartilage. While the differences between the layers comprise the largest variations in the cartilage, they are however not the only source of variation because there are also subtle variations in matrix composition in the regions between chondrocytes(29).

Interestingly, although the amount water and aggrecan vary with depth in the cartilage, the relative amounts of the major collagens (Collagen II, IX and XI) do not(37). However, studies have shown that while the collagens may remain constant throughout the different layers, they do not remain constant over time. During development for example, collagen IX and collagen XI each account for about 10% of the collagens present while collagen II accounts for about 80%. In adult cartilage however, levels of collagen IX and XI decrease to 1% and 3% respectively while collagen II levels increase to almost 90%(37).

1.3.5.3 Intercellular ECM Zones

In addition to the superficial-to-deep organization of cartilage there is also a finer level of organization that occurs within each of the layers. This micro-organization occurs between and around the cells and its boundaries are determined based on the proximity to the cell. This additional level of organization within the layers can be divided into 3 distinct regions: pericellular (the region immediately surrounding the cell), territorial (the region immediately outside the pericellular region), and interterritorial (the large expanses of ECM that exists between adjacent territorial regions). Research has shown that both collagen and aggrecan vary significantly between these regions(38). The pericellular region, which is primarily composed of collagen VI, forms an almost transparent halo around the chondrocytes due to the organization of the fibers(39). In contrast, the interterritorial region is predominantly composed of collagen II and displays the classic arrangement of collagen fibers and aggrecan that is typically associated with hyaline cartilage. Although it remains poorly

characterized, the territorial region appears to be a transition zone which is intermediate in composition between the cell-associated pericellular region and the vast sections of interterritorial cartilage.

1.3.5.3.1 Proteoglycans

In addition to facilitating articulation of the long bones, AC also functions as a shock absorber for the bone. This can occur because water, which is the major component of the synovial fluid, is attracted to the negatively charged proteoglycans that comprise the articular cartilage(40). During compression, water that is retained within the network of proteoglycans is forced out into the joint cavity. When the compressive force is removed, the water is reabsorbed by the proteoglycans thus restoring the cartilage to its former structure and composition(40). This dynamic equilibrium contributes to the resiliency of the shock-absorbing cartilage and allows it to effectively serve the joint for more than a century. In addition to serving as a shock absorber and ensuring mechanical form and function, this unique repetitive hydraulic action also renews the mineral and nutrient supply to the chondrocytes with each cycle. The rate at which the water enters and exits the tissue during loading-unloading cycles is depends on the pore size in the ECM(29), which may play an important role in AC disease progression.

Once considered to be static and unchanging, AC is actually remarkably dynamic in its ability to meet the body's needs. Jurvelin *et al*. demonstrated in a canine model that functional loading of joints resulted in elevated proteoglycan content and increased mechanical stiffness in the cartilage(41). Conversely, in a series of experiments using rabbits, it was found that when joints were immobilized there was an associated loss of proteoglycans and thinning of the cartilage(42). Together these studies have demonstrated that cartilage like, most other tissues in the body, is able to adapt to the demands placed on it.

1.3.5.3.1.1 Proteoglycan Structure

Proteoglycans, as their name implies represent a hybrid of protein and sugar moieties. The entire family of these hybrid molecules share the same basic structural organization [reviewed in (40)]. Proteoglycans, which were originally called mucopolysaccharides, consist of a central core protein to which numerous polysaccharide chains known as GAGs (glycosaminoglycans) are attached(43). The GAGs attach to the core protein in a perpendicular orientation within specific domains to form a brush-like structure. GAGs are linked to the protein core via a specific trisaccharide which is composed of two galactose residues and a xylulose residue. This trisaccharide is attached to a serine residue within the core protein through an O-glycosidic bond. An exception to this occurs in some forms of keratan sulfate which are actually linked to asparagine through an N-asparaginyl bond(40).

Glycosaminoglycans chains are composed of long, unbranched repeating disaccharide units which are covalently linked to a central protein core and typically contain sulfated hexosamine and uronic acid(44). Although there are six GAGs in total, the 4 types that are found in cartilage are chondroitin sulfate, keratin sulfate, dermatan sulfate and hyaluronate.(43) Hyaluronate is different from the others; however, because it is neither attached to a central protein core molecule nor sulfated. In addition to these differences, hyaluronate can reach molecular weights of over a million kilodaltons (kDa). Chondroitin sulfate is noteworthy because although it is only 15-20 kDa in size, it is the most abundant GAG in cartilage comprising between 55-90% and has the greatest number of disaccharide repeats(45). Keratin sulfate is the second most abundant GAG, but unlike the 25-30 disaccharide repeats found in chondroitin sulfate, its chains are considerably shorter. GAGs are highly negatively charged molecules which is the result of the carboxyl ($-COO^-$) and/or sulfate ester groups ($O-SO_3^-$)(40).

Proteoglycans can be loosely grouped into 5 broad categories: large extracellular, small leucine-rich, cell-associated, basement membrane-associate and nervous

tissue-associated proteoglycans. The most important proteoglycan families in cartilage are the large extracellular proteoglycans and the small leucine-rich proteoglycans(29). The large extracellular family is comprised of four family members known as aggrecan, brevican, versican and neurocan which are expressed in a tissue specific manner(46). Aggrecan is the cartilage-associated proteoglycan and comprises the majority of all cartilage proteoglycans.

1.3.5.3.1.1.1 Aggrecan

Despite the fact that the aggrecan core protein is over 2000 amino acids in length, almost 90% of the mass of an aggrecan molecule can be attributed to its GAG side chains(47). Although both chondroitin and keratin sulfate are associated with aggrecan, the majority of the GAG molecules are chondroitin sulfate. Aggrecan is comprised of three globular domains (G1, G2 and G3) and the four distinct regions between them known as the interglobular domain, chondroitin sulfate domains 1 and 2 and keratan sulfate domain(44). The G1 and G2 domains are located in the N-terminus of the molecule while G3 domain is located in the C-terminus. The interglobular domain (IGD) resides between the G1 and G2 domains while the remaining three GAG-rich regions reside between G2 and G3 domains. The chondroitin sulfate (CS) domain is the largest of the domains, accommodating approximately 100 separate CS chains(43). In contrast to the extracellular proteoglycans, the small leucine-rich proteoglycans (SLRPs) form a superfamily of more than 12 different proteoglycans that can be subdivided into 3 main groups(48). In cartilage, the most important of the SLRPs are decorin, biglycan and fibromodulin; however, others like lumican have been observed.

1.3.5.3.1.1.2 Decorin

Compared to aggrecan, decorin is a relatively small proteoglycan with a protein core of only 30 kDa(29). While decorin has since been found in a variety of tissues, it is most commonly associated with cartilage and bone. Decorin was so named because it was initially described as a molecule that decorated the collagen matrix. Unlike aggrecan which has numerous GAGs associated with it, decorin

only has a single N-terminal GAG binding site(49). Initially there was confusion over which class of GAG was bound to it, but it is now generally agreed that it can be either a chondroitin or dermatan sulfate chain(50). Decorin, with its horseshoe-shape binding site, is the most abundant of the SLRPs and is believed to play an important role in maintenance of the collagen II triple helical structure and intermolecular collagen II spacing.

1.3.5.3.1.1.3 Biglycan

Like decorin, biglycan is also a small proteoglycan which can be found in a vast array of tissues. Unlike decorin however, biglycan has 2 N-terminal dermatan/chondroitin sulfate binding sites. While there is considerable homology in their core protein (which consists of 12 consecutive 24 amino acid repeats) their genes are located on different chromosomes(45). Despite being very similar, biglycan and decorin are clearly not interchangeable because decorin localizes to the interterritorial region and biglycan localizes to the pericellular region within articular cartilage.

1.3.5.3.1.1.4 Fibromodulin

Unlike decorin and biglycan, fibromodulin is a class II SLRP which is composed of a protein core to which four, centrally located, N-linked keratin sulfate chains are attached(50). While similar in structure to decorin and biglycan, the core protein of fibromodulin is composed of 10 repeats of 23 amino acids and is approximately 50 kDa in size(45). Despite the small size of these SLRPs and that fact they only comprise a small portion of the overall proteoglycans in cartilage, they are almost as numerous in quantity as aggrecan(29).

1.3.5.3.1.1.5 Link Protein

The link protein is a small protein that is homologous to the aggrecan N-terminal G1 and G2 globular domains. The link protein, like the two aggrecan domains is composed of small duplicated loops known as proteoglycan tandem repeats (PTRs). The role of the link protein is to stabilize aggrecan and enable the

formation of an exceedingly stable noncovalent bond between aggrecan and hyaluronan. Both the PTRs in the link protein and G1 domain of aggrecan have been shown to be involved in hyaluronan binding(51). Interestingly however, for reasons that remain unclear, the G2 domain of aggrecan despite its 67 % amino acid identity with PTR region in G1 is totally incapable of binding hyaluronan.

1.3.5.3.1.2 Aggrecan Biosynthesis

Unlike the GAGs which are not encoded by specific genes, the core protein in the aggrecan molecule is transcribed from the aggrecan gene. Newly synthesized aggrecan transcripts are spliced into one of 3 splice variants whereby they exit the nucleus and are translated via ribosomes on the rough endoplasmic reticulum (ER)(43). Within the ER, the addition of GAG chains to the protein core is initiated by xylosyl transferase which adds a molecule of xylose to selected serine residues based on the recognition of a GAG consensus sequence(52). In the Golgi complex, the synthesis of the trisaccharide linker that will eventually hold the GAGs is completed through the action of specific glycosyl transferases(46). The specific repeating disaccharide units that comprise each of the different GAG chains are then initiated and elongated by their respective set of enzymes. In the case of chondroitin sulfate for example, the repeating polymers are synthesized as the result of the combined action of an N-acetylgalactosoaminyl-transferase and a glucuronosyl-transferase. Sulfonation of the hexosamine residues within the elongating GAG chain is achieved by the action of a sulfotrasferase enzyme(53).

When the mature proteoglycans are complete, they are excreted into the ECM where they aggregate with hyaluronan, which is facilitated through the interaction with a link protein. Ultimately numerous aggrecan molecules will bind to hyaluronan in this manner resulting in the formation of enormous macromolecular arrays. In the extracellular environment, these massive proteoglycan complexes along with a host of smaller proteoglycans will interact with and bind to the collagen scaffold resulting in the production of an ECM with the qualities of hyaline cartilage. This simplistic model however, while it may explain

proteoglycan assembly in the pericellular region does not provide an adequate explanation to account for synthesis/maintenance of the proteoglycan meshwork throughout the vast interterritorial expanses. Oegema(54) showed that newly secreted aggrecan was not immediately processed into larger aggregates. However; it was not until more recently that researchers began to understand the nature of the delay. It is now generally accepted that hyaluronan affinity evolves with time as the disulfide bonds mature within the newly synthesized aggrecan. Perhaps more importantly, it has recently been demonstrated by Bayliss *et al*. that aggrecan is processed in distinct pools which display different hyaluronan binding affinities based on their association with the link protein(55). When taken together, this suggests that aggrecan is likely transported through the tiny pores in the ECM as a monomer rather than a completed aggregate with hyaluronan. Interestingly, Bayliss *et al*. also discovered that there was a significant reduction in the rate of aggrecan incorporation with increasing age which offers insight into one mechanism for age- related tissue decline(55).

1.3.5.3.1.3 Aging and Proteoglycans

Due to the critical role that the negatively charged GAGs and hence proteoglycans play in the retention of water within the cartilage matrix, it is conceivable that even small, seemingly insignificant changes could have disastrous consequences on the mechanical properties of AC. Since all tissues show signs of age-associated wear, it should not be surprising to see similar changes in cartilage. Interestingly, it is within some of these essential water-retaining molecules that researchers have found some of the most profound changes. Plaas *et al*. for example discovered that there were significant age-associated differences in the length of the chondroitin sulfate chains(56).

In addition to changes in the length of the GAG chains, there are also age-related changes in their relative proportions. According to Mankin *et al*. keratin sulfate levels are low and chondroitin sulfate levels high in youth but this relationship reverses in the elderly(29). This is significant for two reasons 1) the keratin

sulfate molecules are shorter than the chondroitin sulfate which results in a reduction in the overall amount of GAGs and hence net negative charge within the tissue 2) the shorter keratin sulfate chains would not be able to associate with as extensively with the other ECM components to the same extent resulting in a greater pore size within the matrix and a reduction in structural integrity. It is thought that such age-related changes may play a role in disease development.

1.3.5.3.2 Collagens

Currently, there are over 20 distinct collagen types that have been identified which are expressed in a broad range of different tissue types(57). The different collagen types were each assigned a roman numeral based on the order in which they were discovered(58). Each of these collagen molecules, irrespective of the collagen type, is itself assembled from a combination of 3 of the 26 genetically different collagen α-chains that are now known to exist (57). Although these α-chains are encoded by genes scattered across 14 different human chromosomes, due to a common amino acid motif, all form a characteristic left-handed helix. This motif, which consists of a triplet of amino acids with glycine in every third position (Gly-X-Y) is common to all collagenous molecules. The repeating motifs enable individual helices to combine with one another to form either homo or heterotrimers. Due to the sequence similarity between the α-chains, a unique stereochemistry arises which enables them to interact with each other and coil together around a central axis thus yielding a right-handed superhelical tropocollagen molecule(59). In the triple helical arrangement, the smaller glycine residues, which are staggered by one amino acid in each α-chain, are oriented towards the center of the collagen molecule and allow it to pack together very densely.

These collagen molecules can be grouped into different collagen families according to their supramolecular structure and function (Table 1.3). The fibril forming collagens are by far the most abundant collagen family, comprising almost 90% of all collagen found in the body(57). Although members of this

family can be found in a variety of tissues, they are most commonly associated with bone (collagen I and V) and cartilage (collagen II and XI).

Table 1.3. Collagen families and the respective collagen types that comprise them.(adapted from Gelse *et al*., 2003)(57)

Collagen Family	Collagen Type
Fibril forming collagens	I, II, III, V, XI
Basement membrane collagens	IV
Microfibrillar collagen	VI
Anchoring fibrils	VII
Hexagonal network forming collagens	VIII, X
FACIT collagens	IX, XII, XIV, XIX, XX, XXI
Tramsmembrane collagens	XIII, XVII
Multiplexins	XV, XVI, XVIII

1.3.5.3.2.1 Collagen Structure

Fibril forming collagens such as collagen I, II and III, due to their vast uninterrupted repeating domains, are the longest and strongest of the collagen molecules (~1000 amino acids)(60). In addition to their strength, these long tightly packed molecules which provide the bulk of the collagenous framework within tissues, are also incredibly resistant to enzymatic digestion by pepsin, trypsin or chymotrypsin. As a result, collagen fibers form a network that is very stable and resistant to degradation. The collagen network in human articular cartilage is so stable that researchers like Maroudas have estimated it to have at turnover time of almost 400 years(61).

Unlike the fibril forming collagens however, the other collagen families all have non-collagenous interruptions to varying degrees in their collagenous domains.

These non-collagenous domains, while providing protein binding regions, aggrecanation sites or hinge domains within these collagen molecules, also result in increased enzymatic access to the protein. This more open protein configuration ultimately leads to increased cleavage rates and lower molecular stability(57). These intra-collagenous domain regions are not the only non-collagenous regions that are associated with collagen molecules. Both the amino and carboxy termini of collagens contain non-helical propeptides that form structures known as globular domains. In the immature collagen molecules, known as procollagens, the majority of these propeptides are cleaved leaving behind only tiny non-helical portions known as telopeptides. The amino and carboxyl propeptides in collagens are cleaved by specific enzymes known as N and C-procollagenases respectively. Cleavage of the procollagen by the procollagenases results in the production of a mature tropocollagen molecule. In the case of Type I procollagen for example, which is the most commonly studied, there are an additional 150 amino acids on its N-terminus and an extra 250 amino acids on its C-terminus.

These terminal globular domains are believed to play a very important role in the assembly and maturation of procollagen. During the initial assembly of procollagen, multiple disulfide bonds form within the N-terminal globular domains of the α-chains which serve to stabilize the newly forming complex. In addition to the intra-chain disulfide bonds in the N-terminus, the C-terminus contains numerous inter-chain disulfide bonds which hold the α-chains in close proximity. This close proximity allows for proper hydrogen bonding between the amino acid residues and hence the formation of proper quaternary protein structure(62). Genetic mutations in the α-chains, the absence of chaperone proteins or changes in the physical environment can alter this delicate assembly process and result in the production of a denatured form of collagen resembling gelatin.

1.3.5.3.2.2 Collagen Types in Articular Cartilage

1.3.5.3.2.2.1 Collagen I

Collagen type I is a rigid rod-shaped molecule 30 000 nm in length and 150 nm in diameter that represents the basic functional unit of the collagen fibril(63). It is by far the most abundant of all the collagens and accounts for about 80% of the collagen found in the body. It is found in a broad range of tissues that include bone, skin, tendon ligament, uterus, cornea(64) and is routinely the collagen to which all other collagens are compared. Collagen type I is a heterotrimer because it is composed of two α-1(I) chains and one α-2(I) chain. Defects in collagen type can have very serious consequences and lead to a variety of conditions including: osteogenesis imperfectae, Ehlers-Danlos syndrome as well as a range of degenerative disorders(65). Although not normally found in healthy cartilage, its expression has been associated with *in vitro* growth of chondrocytes(6,18,66).

1.3.5.3.2.2.2 Collagen II

Although collagen type II is typically associated cartilaginous tissues, it does occur within other tissues in the body including: nucleolus pulposus and the vitreous humor. In contrast to collagen type I, collagen type II is a homotrimer which is composed of three identical α-1(II) chains. Interestingly, two different isoforms of this α chain exist which are expressed at different stages of development. When cartilage first begins to develop during embryogenesis, a special mRNA splice variant (IIA) containing exon 2 of the gene is expressed. Throughout adulthood; however, IIB is the predominant isoform, and it lacks the additional exon that encodes for a cystein-rich N-terminal globular domain(57). Experimentally, this switch in splice variants yields a useful biomarker in the chondrogenesis pathway from undifferentiated stem cell to fully differentiated chondrocyte. In addition to being expressed during embryonic development, occasionally the IIA splice variant is found in chondrosarcomas.

1.3.5.3.2.2.3 Collagen III

Like collagen type II, collagen type III is also composed of a homotrimer of α-1(III) chains, but unlike collagen type II, it is quite ubiquitous and not associated with any specific type of tissue. Although it is the second most abundant collagen in skin, rather than being known for a tissue specific association, collagen type III is better known for it physical properties. Collagen type III, unlike the other fibril-forming collagens, is associated with tissue extensibility and hence found in a number of tissues that rely on elasticity such as lung, heart, nerve, spleen, kidney and cartilage(58).

1.3.5.3.2.2.4 Collagen VI

Collagen type VI is a microfibrillar collagen and as such is in a class of collagens all on its own. This type of collagen is a heterotrimer but unlike type I it is comprised of 3 different α chains [α-1(VI), α-2(VI) α-3(VI)]. Its α chains, in addition to showing considerable inter-chain variability, also have very short helical domains which are bounded by unusually large globular domains(67). Collagen VI α chains also display numerous splice variants and their gene products are subjected to an extensive array of post translational modifications, making them one of the most variable of the collagens. Collagen VI is found in relatively low levels in almost all tissues including cartilage.

1.3.5.3.2.2.5 Collagen IX, XII and XIV

In contrast to the other collagens that are found in articular cartilage, collagen IX, XII and XIV belong to the family of FACIT (Fibril-Associated Collagens with Interrupted Triple helices) collagens. As their name implies, these collagens are all characterized by short non helical interruptions in their classical collagenous domains. Like collagen type VI, collagen type IX is also composed of 3 distinct α chains [α-1(IX), α-2(IX) α-3(IX)]. Collagen IX which is the second most abundant collagen in articular cartilage is co-distrubuted with collagen type II. Collagen type IX has been shown to covalently bind to the N-terminal region of collagen type II telopeptide but is also believed to act as an anchor for

proteoglycans(57). Little is known about collagen types XII and XIV but they are thought to play a similar role to collagen IX due to their high degree of sequence and structural homology.

1.3.5.3.2.2.6 Collagen X

Collagen X, which is a homotrimeric molecule composed of three identical α-1(X) chains, is a member of the hexagonal network forming collagen family. This is the most specialized of the collagens because it is restricted in expression to chondrocytes in the hypertrophic region of cartilage that are undergoing mineralization. Because it is such a characteristic molecule in this region it can serve as a very effective biomarker for cartilage calcification. Interestingly, genetic defects in collagen type X have been linked with a disease known as Schmid type metaphyseal chondroplasia (SMCD) where it yields skeletal deformities and shortened limbs.

1.3.5.3.2.2.7 Collagen XI

Unlike some of the fibril-forming collagens which are present in abundance in the tissues that they are found, collagen type XI is only present at relatively low levels and is almost exclusively found within cartilage. Like collagen IX, collagen XI also co-distributes with collagen II within cartilaginous tissue. While it remains poorly understood, it is believed that collagen XI may localize to the central core of collagen II fibrils(57). Collagen XI is a heterotrimer that is comprised of three different α chains, one of which [α-2(XI)] is encoded by the same gene as α-1(II)(60).

1.3.5.3.2.3 Collagen II Biosynthesis

It is generally accepted that the upstream effector of collagen II transcription is the SOX-9 transcription factor. In addition to transcriptionally activating a host of other ECM-related genes, SOX-9 will initiate transcription of the collagen II gene that is located on the long arm of chromosome 12. When the transcript is complete, it is spliced into one of two variants (IIA or IIB) and receives a poly-A

tail to generate the mature transcript. Mature collagen II transcripts then exit the nucleus and are bound by ribosomes embedded in the endoplasmic reticulum (ER). Once translated, a signal peptide is then cleaved and the newly synthesized procollagen is released into the lumen of the ER. In the lumen the procollagen will undergo extensive post-translational processing that will include the hydroxylation of selected proline and lysine residues. These hydroxylation reactions are conducted by a family of hydroxylase enzymes (prolyl 3-hydroxylase, prolyl 4-hydroxylase and lysly hydroxylase) that are dependant on ferrous ions, 2-oxogluterate, molecular oxygen and ascorbate as cofactors(57). A second round of modifications occurs as small carbohydrate molecules are transferred onto the newly created hydroxylysines(57). In addition to accepting these carbohydrates, the hydroxylysine residues will subsequently play a very important role in crosslinking the collagen molecules together. Before that can happen, however, numerous intra-chain disulfide bonds will form in the N-terminal region of the α chain propeptides that will help stabilize each of the chains. As the 3 chains that will form the trimer come together, inter-chain disulfide bonds are formed by protein disulfide isomerase in the C-terminal propeptide which will hold the chains in close proximity for the next phase of assembly. Formation of the triple helix is then initiated in the C-terminus and proceeds towards the N-terminus with the help of collagen-specific chaperone proteins known as HSP47(68).

1.3.5.3.2.4 Collagen II Secretion and Fibril Formation

The completed procollagen is packaged by the Golgi into secretory vesicles for subsequent extracellular release. Once released, specific metalloproteinases known as procollagen N-proteinase and procollagen C-proteinase cleave the N and C-terminal propeptides from the procollagen to yield mature tropocollagen II(69). Interestingly, it is believed that collagen II expression is regulated though the production of these cleaved propeptides through a complex feedback loop. Horlein *et al*. demonstrated that collagen I translation could be specifically inhibited by the addition of propeptides to fibroblast cultures(70). Due to the

similarity between the collagens and their propeptides it is quite likely that collagen II propeptides function in a similar regulatory manner.

While it remains poorly understood, the C-propeptides are also believed to be involved in collagen fibril assembly(57). Although the natural charge distribution and stereochemistry of the fibril-forming collagens allows for a certain degree of spontaneous fibril assembly, the propeptides and their cleavage likely determine aspects such as fibril diameter and morphology. The mechanical resilience of the fibrils arises in large part due to the cross-linking that occurs between hydroxylated lysine residues in the telopeptide region of the molecule. Not surprisingly, cartilage which is the most resilient of the collagenous tissues, has the highest degree of lysine hydroxylation and, hence, cross-links seen in any tissue(60). Although the chemical cross-linking reactions are quite complex, they are initiated by and dependant upon an enzyme known as lysyl oxidase which converts the hydroxylated lysine residues into chemically reactive aldehyde groups. Once initiated, a series of spontaneous reactions will proceed that ultimately result in the formation of a stable cross-link between the telopeptide of one collagen II molecule and the helical domain of an adjacent collagen II molecule(60).

1.3.5.3.3 Chondrocytes

In the developing embryo chondrocytes are abundant in both number and distribution throughout the body because they synthesize the cartilage that forms the precursors to a wide range of skeletal components. However, in the adult, chondrocytes and the cartilage that they maintain are predominantly restricted to the ears, nose, intervertebral disks, larynx, epiglottis, portions of the thoracic cage, and the ends of the long bones.

1.3.5.3.3.1 Differentiation and Chondrogenesis

Although chondrogenesis has been extensively studied as it applies to endochondral ossification, the details surrounding the development of articular

cartilage are not well defined. The development of articular cartilage begins during embryogenesis in a joint like the knee, when chondroprogenitor cells that reside within the future joint space dedifferentiate into mesenchymal cells and undergo apoptosis. This step, which is known as cavitation creates the necessary joint space and leaves the space lined with a layer of chondroprogenitor cells(71). The chondroprogenitor cells then differentiate into chondroblasts that divide rapidly and begin to produce and secrete ECM molecules, thus giving rise to a very thin layer of articular cartilage-like tissue. At about the time of birth the chondroblasts continue to secrete ECM components but gradually lose their replicative potential and mature into fully differentiated chondrocytes(71). Although the rounded cell that resides in adult articular cartilage were traditionally regarded as terminally differentiated, there are some researchers that now refer to this cell type as "maturationally arrested" rather than terminally differentiated(72). The argument for this change in terminology is that if given the appropriate growth cues the chondrocytes will continue down a differentiation pathway towards the type of hypertrophic chondrocyte found below the tide mark and often associated with osteoarthritis.

The genetic expression profile of hypertrophic cells; however, is very different from that found in mature chondrocytes because the expression of collagen X increases while the classic chondrocytic markers like collagen II and aggrecan decrease. This is not merely a change in expression of a few genes but an entire signaling pathway. Activation of the hypertrophic expression profile is mediated by the Cbfa1 transcription factor rather than SOX-9 and suggests that an entirely different differentiation pathway has been initiated. Interestingly, some researchers have actually proposed that hypertrophic chondrocytes may actually have the ability to undergo phenotypic transformation into osteoblasts, which would explain the pronounced phenotypic shift and simultaneously support the former view of chondrocytes as terminally differentiated cells(73). Regardless of the view that ultimately prevails, it should be clear that chondrocytes and their progenitor cells represent a very malleable cell lineage, the fate of which can

likely be influenced by a very broad array of factors. For example, some very recent findings suggest that hypoxic conditions alone may be sufficient to promote the differentiation of mesenchymal cells down a chondrogenic rather than an osteogenic pathway(74).

1.3.5.3.3.2 Subcellular Adaptations

Unlike other tissues which are usually home to a complex array of cell types, chondrocytes are the only cell type found in adult cartilage. As such, chondrocytes are the only cells responsible for producing and maintaining the extracellular matrix that surrounds them. What makes this task even more impressive is that, due to the lack of vascularization in cartilage, they must accomplish this in an oxygen and nutrient poor environment. Because cartilage lacks vascularization, the chondrocytes must rely on the diffusion of the synovial fluid alone to supply them with all the life sustaining minerals and nutrients they need to perform their function. Due to their relatively deprived existence, chondrocytes are well adapted to this environment and their metabolism as well as their cellular physiology reflects this. Because they exist in a hypoxic environment, they are forced to derive their energy through anaerobic respiration rather than the aerobic respiration utilized by most cell types(29). Since they rely predominantly on glycolysis for their energy production rather than oxidative phosphorylation, they have little need for mitochondria and hence have very few per cell(75). Despite the limitations placed on them by their environment, chondrocytes have surprisingly high metabolic rates because they are constantly required to manufacture and remodel their ECM(76).

A decreased number of mitochondria are not the only adaptation that chondrocytes possess to cope with the unique requirements that are placed on them. Because the sole function of a chondrocyte is to produce the collagens and proteoglycans that sustain their ECM, the intracellular machinery has also evolved to serve this function. As a result, chondrocytes have developed a highly elaborate endoplasmic reticulum and an extensive Golgi apparatus to

accommodate all of the protein synthesis that is required to produce such a vast amount of ECM(44).

1.3.5.3.3.3 Molecular Adaptations

In addition to alterations in the size and distribution of their organelles, chondrocytes have also had to adapt to their environment and the related demands that it places on them. Beyond the range of cartilage-associated ECM genes that are expressed by chondrocytes, the regulation of those genes is also unique. A family of heterodimeric transcription factors known as hypoxia-inducible factor (HIF) for example, has gained much attention for its ability to up-regulate a host of specific genes in response to declining oxygen availability. Recently the hypoxia inducible factor-1 (HIF-1) has been implicated as a central transcription factor in the process of chondrogenesis(74). Reports indicate that HIF-1 is also involved in a diverse array of essential chondrocyte processes including: extracellular matrix (ECM) synthesis(77), cell proliferation(78), apoptosis(79) and even glycolysis(80). Interestingly, when oxygen availability is low, HIF-1 has been shown to transcriptionally activate a series of genes resulting in the switch from an aerobic mode of metabolism to a glycolytic mode of metabolism (81).

HIF-1 is a heterodimeric complex consisting of a constitutively expressed oxygen sensitive subunit known as HIF-1_{α} and a nuclear translocation factor known as HIF-1_{β}(82). Under low oxygen tension, HIF-1_{α} translocates into the nucleus, associates with HIF-1_{β} and binds to short DNA sequences known as hypoxia responsive elements (HRE)(83). Although HREs have been identified in a variety of genes including: erythropoietin(84), transferrin(85), vascular endothelial growth factor(86), and glucose transporter-3(87), it remains unclear whether ECM genes such as collagen II and aggrecan are directly transcriptionally activated by HIF-1. Another transcription factor known as SOX-9, which also plays a pivotal role in chondrogenesis, may represent a molecular signaling intermediate between the oxygen sensing of HIF-1 and the transcriptional activation of ECM genes in chondrocytes. The *SOX-9* gene, which is transcriptionally activated by HIF-1(74),

has itself been previously shown to be involved in the transcriptional activation of genes such as collagens II, IX and, XI as well as aggrecan(88) and hence represents an excellent potential downstream modulator in HIF-1 induced maintenance of the chondrocyte phenotype.

1.3.5.3.3.4 Mechanotransduction

Another type of signaling molecule that plays an important role in chondrocytes is the integrin family and the role these attachment molecules have has been gaining more attention in recent years. Integrins, which are heterodimeric transmembrane glycolproteins and comprised of α and β subunits, were originally implicated in cell anchorage. In addition to anchoring the actin cytoskeleton to the extracellular matrix, it is now clear that integrins play another important role. Currently there are 16 α subunits and 8 β subunits that are known to exist, which can combine to form more than 20 distinct integrin molecules(11). Each of these integrins acts as a specific membrane-bound receptor for a different component of the extracellular matrix. The important integrins in cartilage are the α1β1, α2β1 and the α11β1 which all are known to bind collagen II and believed to play a role in maintaining the differentiated phenotype(11). Because integrins span the plasma membrane and interact with elements of both the cytoskeleton and the ECM, they can transduce external mechanical stimuli (like shear forces via the α5β1 integrin) into internal biochemical stimuli that can bring about changes in gene expression.

Although the mechanism underlying the observation was unclear at the time, studies in both 2D and 3D cultures showed that when mechanical stimulation was provided to chondrocytes, there was a pronounced increase in both aggrecan and collagen II synthesis(11). Armed with this new view of integrins as signal transduction molecules involved in the regulation and expression of extracellular components, as well as the maintenance of the chondrocytic phenotype. Brodkin *et al*. examined the nature of integrin binding on ECM expression, cell morphology and the differentiation status of chondrocytes. Due to prior studies that had shown that 3D culture, which favored the round chondrocyte

morphology, could preserve the differentiated chondrocytic phenotype(5,89), Brodkin *et al*. believed that if they could satisfy the cells' integrin binding requirements and hence trigger appropriate intracellular chondrogenic signaling, that they would be able to maintain fully differentiated chondrocytes in 2D culture(6). Unfortunately their study was inconclusive because they failed to fully explore and understand the variables in the model system that they were employing (ie. cell density, 3D culture requirement and long duration culture). As an example, Watt has shown that high cell density culturing, which likely activates and satisfies a large portion of cellular integrins, is a very important factor in maintaining the differentiated chondrocytic phenotype(13). Cell density also plays an important role because integrin activation leads to paracrine signaling and tissue-wide responses(90). With the low cell densities in the above experiments, it is possible that either an integrin binding threshold or an integrin signaling threshold was not attained which could have permitted the maintenance of the fully differentiated chondrocyte phenotype. The intricacies of the signal transduction pathways downstream of integrins remains poorly understood, but it is likely that SOX-9 is a downstream effector for the collagen II binding integrins.

1.3.5.3.4 Matrix Metalloproteinases

In addition to the extracellular proteins, attachment complexes and signaling molecules discussed above, chondrocytes also express a range of ECM degrading enzymes and their associated protein inhibitors. Matrix Metalloproteases (MMPs) represent a 20-member multigene family of zinc containing, Ca^{2+} dependent endopeptidases that can be grouped into subfamilies based on substrate recognition(91,92) (Table 1.4).

Of all of the MMPs, the collagenases (MMP-1, 8 and 13) are able to recognize and specifically cleave the vast majority of all collagens in their native fibrilar form. The gelatinases (MMP-2 and 9) are also able to recognize and cleave some of the collagens but they are incapable of catalyzing the cleavage reaction on undenatured collagens and are thus dependent on the true collagenases to make

the initial helix-denaturing cut(93). While most proteinases will recognize multiple cleavage sites within their substrate molecule, MMPs make a single cut resulting in characteristic fragments which represent ¼ and ¾ of the size of the initial substrate molecule(93).

Table 1.4. Matrix metalloproteinases families and their respective MMPs. (adapted from Thomas *et al*., 1999)(92)

Collagenase	Gelatinase	Stomelysin	Membrane-Type	Matrilysin
MMP-1	MMP-2	MMP-3	MMP-14	MMP-7
MMP-8	MMP-9	MMP-10	MMP-15	
MMP-13		MMP-11	MMP-16	
MMP-18		MMP-12	MMP-17	
		MMP-19		
		MMP20		

Despite the overlap in the collagen substrates between the different collagenases, MMP-13 was shown to have the highest affinity for the cartilage-associated collagen II(94). The third major class of MMPs, known as stromelysins (MMP-3, 10 and 11), have a very broad substrate specificity and are able to degrade collagens, proteoglycans, gelatins and even fibronectin. A recently discovered fourth class of MMPs is the membrane-type MMPs which are bound to the cell membrane through their transmembrane domains.

1.3.5.3.4.1 MMP Regulation

MMP synthesis and activity are very tightly regulated within chondrocytes which is necessary if these destructive enzymes are to be prevented from inadvertently causing tissue-wide degradation. For this reason, MMPs are expressed as inactive proenzymes which require the cleavage of a regulatory cysteine residue to becoming activated. Once activated, the activity of the MMP can still be blocked by a class of MMP regulatory molecules known as tissue inhibitors of MMPs (TIMPs) which function by binding tightly to the active site of the MMP(93). A novel cell-mediated regulatory mechanism for MMP activation may involve MT-

MMPs, because they have been found to form trimolecular complexes with MMPs and TIMPs. In addition to the MMPs, another family of over 20 metalloproteases known as ADAMTSs was recently discovered(95). Beyond simply cleaving some of the collagens, this new family of proteases was also shown to cleave aggrecan.

1.3.5.3.4.2 ADAMTS

While ADAMTS-2 and 3 were found to be specific for collagens, studies have revealed that ADAMTS-1, 4 and 5 display the characteristic aggrecan cleavage pattern that had been previously attributed to the as yet undiscovered aggrecanases(93). Although little is known about the details of their regulation, their activity has long been associated with tissue inflammation. As such it was not surprising when it was discovered that IL-1 plays a major role in their up-regulation. Despite sequence similarities with the MMPs, most TIMPs are unable to inhibit ADAMTS. The exception seems to be TIMP-3 which was recently shown to be capable of inhibiting ADAMTS-4 and 5(96).

1.3.5.4 Osteoarthritis

There are currently more than 100 different medical conditions that fall under the category of arthritis. The two most common are osteoarthritis (which is age-related) and rheumatoid arthritis (which is an autoimmune-related). Osteoarthritis (OA) is the most common type of arthritis and the number one cause of disability worldwide. It is a degenerative joint disease that characterized by joint pain and dysfunction ultimately resulting from joint deterioration. Currently there are no known methods for either preventing the disease or even slowing its progression, which is why treatment for OA is often considered merely palliative in nature. It is estimated that OA negatively impacts the quality of life of over 20 million Americans and costs the US government in excess of 60 billion dollars per year(97). Moreover, because OA is predominantly a disease of the elderly, as our populations ages, incidences of the disease as well as the associated costs are predicted to soar over the next two decades(98).

1.3.5.4.1 Clinical Presentation

Clinically, a diagnosis of OA is usually established based on chronic joint pain, stiffness and radiographic evidence of a reduction in cartilage volume. Commonly associated with a reduction in joint space is the appearance of osteophytes (aka bone spurs), bone cysts and an increase in subchondral bone density. In more advanced cases of OA, it is not uncommon to find joint dysfunction, joint contractures, muscle atrophy and even limb deformity(97). Primary OA, which is the most common form, develops in the absence of a known cause and almost exclusively occurs in patients over the age of 40(99).

In contrast, secondary OA develops as the result of a preexisting condition which may include one or more of the following: physical joint injury, hereditary/inflammatory disease or developmental, metabolic or neurologic disorders. Although OA affects all ethnic groups and occurs in both genders, it is known to strike with different prevalences in these groups. For example, studies have shown that North Americans have a higher incidence of OA than Asians and the disease occurs more commonly in women than in men(100). The progression of OA can also be highly variable, often with alternating periods of destruction, remodeling and attempted repair of the cartilage(29).

1.3.5.4.2 Histology

The gross appearance of OA in an arthritic knee can vary significantly from patient to patient. In addition to the occurrence of a variety of different lacerations, fissures and ulcers on the surface of the AC, it is also common for there to be yellowish discoloration and an obvious softening of the tissue(101). Interestingly, in a number of OA cases, it is possible to observe tiny, pebble-sized regions of *de novo* cartilage synthesis immediately adjacent to some of the most pronounced damage. This *de novo* synthesis of cartilage is also commonly associated with osteophytes when they are present(57). In more advanced OA cases the ulcerations often become so pronounced that large sections of bone are revealed which often have a very polished appearance. At the microscopic level,

the first signs of OA often include the appearance of fissures that deepen with the progression of the disease. As the fissures deepen, horizontal cracks in the cartilage begin to appear. Another early sign of OA is the loss of proteoglycan staining in the superficial layers, but in more advanced cases the entire matrix can become virtually devoid of staining(101) (Fig. 1.3).

Figure 1.3: Histologic section of OA cartilage stained with hematoxylin and eosin illustrating the fissures, fibrillation and cellular clustering commonly associated with the disease.

1.3.5.4.3 Risk Factors

Since there is currently no cure available for OA, understanding and minimizing the risks associated with the development and progression of the disease is paramount. While age is undeniably the largest of the risk factors, it is important to realize that normal joint use over a lifetime does not cause the cartilage degeneration associated with OA(97). The precise role of aging in the development of OA remains unclear. However; it is generally believed that aging increases the risk by gradually and systematically undermining the ability of the chondrocytes to maintain and repair the cartilage(102). Furthermore, with age, chondrocytes become less responsive to the growth factors and mechanical stimuli that normally promote ECM production. In addition to cellular changes in the cartilage, it is believed that ligaments loosen with age, thus adversely affecting joint biomechanics and increasing the rate of cartilage wear(103). The increased wear and reduced cellular ability to respond combine to significantly increase the risk.

1.3.5.4.3.1 Obesity

Of all of the preventable risk factors influencing the development or progression of OA, obesity is without a doubt the one with the largest impact(104). According to Felson *et al*. women that lost an average of 11 lbs had an associated 50% reduction in the risk of developing OA(105). Not surprisingly, obesity has also been shown to be a risk factor in the progression of the OA(104). Although obesity certainly plays a role in joint loading, it is not clear whether the elevated risk associated with the additional weight on the joint or other more complex biochemical factors are responsible.

1.3.5.4.3.2 Diet

Studies that examined vitamin C intake discovered that individuals who took high levels of daily supplements were at a 3-fold lower risk of developing OA than those that consumed lower levels of vitamin C(106). Although it was thought that vitamin C provided protective antioxidant effects at the cellular level, other antioxidants like vitamin E and β-carotene had no effect. Given the link between vitamin C and collagen II synthesis, it is possible that the elevated vitamin C is actually promoting ECM production rather than providing a protective cellular effect against antioxidants. Due to its role in bone metabolism vitamin D has also been examined. In a related study, it was found that the risk for disease progression rather than onset was increased 3-fold for individuals with low vitamin D intake(107).

1.3.5.4.3.3 Joint Loading

In addition to ligaments that loosen with age like those involved in varus and valgus laxity, other pre-existing conditions can adversely affect the way in which a joint is loaded. Not surprisingly, abnormal joint alignment is likely to represent a significant risk factor for the development and progression of OA(105). Proprioreception, which is used to monitor the movement of a limb through space, is another factor that has also been implicated as a risk factor in the development of OA(105). Abnormal, prolonged or excessive joint loading have also proven to

be important risk factors in the development of OA. A study by Zhang *et al*. revealed that squatting for as little a 2 hrs per day, which is not uncommon in a number of trades, can account for a dramatic rise in the incidence of OA in those groups(108).

1.3.5.4.3.4 Gender

Given the elevated level of OA in women, there is a possibility that estrogen plays a role in the development of OA and hence could be considered a risk factor(103). Studies in this area however have proven inconclusive(109).

1.3.5.4.3.5 Genetics

Despite the heterogeneity with OA, there seems to be a pronounced genetic component to the disease. According to Spector *et al.* between 39-65% of that variability in OA cases can be attributed to genetic factors(110). A number of potential candidate genes exist to explain the strong genetic component of OA and they include: vitamin D receptor, Insulin-like growth factor (IGF-1) and cartilage oligomeric protein (COMP). Furthermore, recent genetic analyses have identified regions on chromosomes 2q and 11q that appear to be involved in OA(105).

1.3.5.5 Age-Related Alterations

Perhaps one of the earliest and most prominent macroscopic changes visible in cartilage is the development of tiny, fingerlike projections (a process known as fibrillation). Although it is often seen in conjunction with OA, studies on autopsy-derived normal AC have shown this to be a common age-associated change in AC(111). In support of this observation, individuals showing signs of articular cartilage fibrillation do not suffer any joint pain or dysfunction as a result(111). While it likely represents a sign of aberrant ECM regulation, and hence could be a predisposing factor for the development of OA, it not a result of the disease process itself.

Arguably one of the most profound age-related changes that are seen in cartilage occurs at the level of the proteoglycans. At the macromolecular level, electron microscopy studies have shown that there is a reduction in the size of proteoglycan aggregates with increasing age(99). Not only is there a reduction in the size of the aggregates, but here are also numerous changes that occur in the aggrecan molecules that compose them(99). In addition to the differences in size of the aggregates there are also differences in the rates of formation of the aggregates, because Bayliss *et al.* have shown that the rate of aggregate formation is much higher in young cartilage than old cartilage(55). There are also age-related changes in the size of the aggrecan core protein(99,112); however, it remains unclear whether these changes stem from post translational alterations or enzymatic cleavage. Finally, age-related changes have also been discovered in the length and abundance of the chondroitin sulfate chains that form aggrecan molecules(113). Lauder *et al.* have shown that the amount of KS increases with age in bovine articular cartilage(114). In addition to structural changes in the GAG chains, Hickery *et al.* have also shown that biosynthesis rates can also be adversely affected with increasing age(115). They demonstrated that chondrocytes show an age-related reduction in responsiveness to TGF-β-induction of GAG synthesis. Initial changes and/or reduction in the cartilage proteoglycans is believed to have profound effect on the water content in the cartilage(111). It is believed that any reduction of water could adversely affect the load bearing properties of the cartilage by increasing the coefficient of friction. In return, an increase in friction may increase the rate of wear on the cartilage thus initiating a degenerative cycle which could ultimately culminate in the development of OA.

Despite the fact that collagens are inherently more resistant to degradation than proteoglycans, they have been gaining attention as researchers attempt to unravel the mystery of age-associated changes in cartilage. In a study looking at the effects of aging on collagen II, Hollander *et al.* showed that up to age 41 there was virtually no signs of collagen denaturation(116). After age 41 however, there was an age-related increase in the amount of collagen II denaturation in the

superficial layer which was also more pronounced in the pericellular region immediately surrounding the cells. They also noticed that in normal individuals showing signs of fibrillation, the amount and depth of denaturation in the collagen II was more pronounced. When AC from patients suffering with OA was examined, it was found that the loss was even more pronounced, which again suggested a link between age, fibrillation status and OA. In a related study, Hollander *et al.* found almost a six-fold increase in denatured collagen II in OA samples when compared to normal controls. This suggested that although collagen degradation may not be an early event in either cartilage aging or OA, it is an important one(117). A study which examined the rates of production of collagen propeptide levels, found that as a result of the loss of collagen II, chondrocytes attempt to compensate by increasing rates of collagen II synthesis(117). Although chondrocytes clearly attempt to restore ECM homeostasis it is possible that the loss of collagen II may represent the first irreversible step leading towards OA. Rates of synthesis and degradation of collagen II may be some of the most obvious changes but they are not the only ones. Verzijl *et al.* have shown that the collagen II network increases in stiffness with age as the result of crosslinks that are formed through a process known as glycation(118). Stiffening may play an important role in the aging of cartilage because it alters the mechanical force distribution within the tissue. This type of change could account for increased wear, alterations in the hydraulic properties or even changes in the cellular responses to mechanical stimuli. Although there is ample evidence of age-related changes in the ECM, there is as yet little or no clear evidence that any one of these changes plays a direct role in the development of OA(101).

1.3.5.6 Molecular Alterations

Studies over that past couple of decades indicate that chondrocytes, like so many other cell types in the body, begin to show age-related declines in a variety of cellular functions[reviewed in (111,119). It is a widely held belief that these age-related declines, at least in other tissues, seem to result from the accumulation of

oxidative damage which after several decades leaves a large proportion of the cells within a tissue incapable of performing their basic function(76,120). In the case of chondrocytes, their function is to maintain the ECM which provides an almost frictionless surface on which the long bones can articulate. Failure to maintain the quality of the ECM ultimately allows the normally well tolerated shear forces within the joint to mechanically and hydraulically tear the cartilage apart. The findings that chondrocytes from elderly donors have been shown to synthesize smaller aggrecans with fewer and shorter chondroitin sulfate chains as well as display less functional link proteins(97) appears to be consistent with this view.

1.3.5.7 Cartilage Degradation

A variety of MMPs (MMP-1, 2, 3, 8, 9, 13 and 14) are found in human articular cartilage but the primary MMPs that are known to be over-expressed there are MMP-3, 13 and 14(121). Perhaps more important than their relative expression levels is that MMP-3, 13 and 14 have also recently been implicated in the OA-related breakdown of cartilage(122). MMPs were initially assumed to be the major culprits in cartilage deterioration. However; new evidence is emerging that supports the aggrecanases (ADAMTS-4 and 5) in that role [reviewed in (123)]. Studies using 3D-based culture systems have shown that when induced with IL-1, aggrecanases are the initial enzymes involved in cartilage digestion but that MMP activity soon follows(124,125). Given that MMPs are not exceedingly large molecules, it is possible that the native density of healthy cartilage is such that MMPs are not granted access to their respective cleavage sites until the aggrecanases have relaxed the ECM. The development of OA may therefore depend on the sequential action of the aggrecanases and collagenases. It is believed that under these conditions significant and potentially irreversible collagen II denaturation and digestion may occur which will eventually lead to OA if left unchecked(23). As the ECM begins to break down structurally the collagen crosslinks that are lost resulting in an increase in pore size within the matrix and an associated reduction in the tissue's ability to resist high joint loads.

A decrease in the ability to cope with the intense hydraulic and mechanical pressure may be an early event leading to OA.

The regulation of the ADAMTS family of metalloproteinases remains unclear, but evidence has emerged that suggests that it may occur through post-translational mechanisms. This explanation was supported by experiments in which ADAMTS activity was induced by cytokines (IL-1, and TNFα) in a cartilage explant model without an associated increase in mRNA(126). While contrary reports in the literature exist, they were all performed in 2D culture systems which may not accurately reflect the mechanism for the *in vivo* mode of regulation.

Cartilage is not the only source of degradative enzymes, because a host of MMPs including MMP-13 (which is predominantly expressed in cartilage) have been found to be expressed by the synovium. Studies with MMP-13 for example have shown that the levels expressed in OA, while elevated, were only one fifth of the levels found in the synovial of RA patients(127). Although the synovium can clearly express these enzymes, the bulk of MMPs are produced by the cartilage(36).

1.3.5.8 Cytokines

Perhaps more important than the proteases themselves are the upstream cytokines that initiate the catabolic cascade ultimately responsible for their activation. There are over a dozen different cytokines that are involved in cartilage metabolism. These cytokines can be grouped into one of four categories based on their function(128). Aside from the obvious role of catabolic and anabolic cytokines, two other categories can be identified: modulatory and anti-catabolic. Modulatory cytokines temper or regulate the actions of other cytokines while anti-catabolic cytokines directly inhibit/antagonize the catabolic cytokines. The most extensively studied cytokines in cartilage are: IL-1, TNF-α, IGF-1, TGF-β and the BMPs. IL-1, which is the prototypical pro-inflammatory cytokine, has been widely studied for its role in cartilage metabolism and OA. Early observations

showed that in monolayer culture IL-1 had the ability to elicit the production of proteases from chondrocytes(129). More recently, the expression of MMP-1 and MMP-13 in chondrocytes has shown a significant increase in response to IL-1, thus directly implicating IL-1 as a factor in collagen II degradation(130). IL-1 effects are not restricted to collagen II however, because recent studies by Hickery *et al.* have shown that it can also dramatically reduce the rate of GAG synthesis(115). Interestingly, the Hickery study also shed light on another facet of cytokine involvement in OA. Anabolic cytokines like TGF-β for example, have long been known to stimulate ECM production in cartilage (specifically collagen II and aggrecan), but more recently it has been shown that chondrocytes become less responsive to these anabolic cytokines with age. Hickery also demonstrated that GAG synthesis is adversely affected by this age-related reduction in responsiveness to TGF-β. While this reduction in responsiveness is only associated with age at the moment, it may ultimately account for the reduced ability of chondrocytes to respond to the demands placed on them during disease progression.

The bulk of IL-1 production likely arises from the synovial membrane; however, chondrocytes produce an IL-1 precursor which is not secreted. Mounting evidence suggests that this immature IL-1 may play an intracellular role in the expression of pro-inflammatory genes(128).

1.3.5.9 Model Systems

Given the exceedingly complex role that the ECM plays in regulating enzyme access, sequestering various proteins and satisfying the attachment requirements of chondrocytes, Caterson *et al.* makes a very strong argument for the use of 3D explants rather than 2D cultures for cartilage degradation research(23). Failure to employ an appropriate exlant model system under these circumstances will almost certainly lead to erroneous or artifactual results. Evidence in support of this comes from the simple observation that chondrocytes in 2D culture adopt a completely different, fibroblastic, phenotype(19).

For studies not specifically requiring a highly developed ECM, the use of articular cartilage is often impractical and cell culture-based alternatives have been examined. Although it has long been known that chondrocytes in monolayer culture lose their phenotype(19) considerable effort has been made to circumvent this problem. Over the past three decades, a range of culture conditions have been developed in an attempt to prevent the *in vitro* dedifferentiation of chondrocytes. Studies have assessed the phenotypic stability of chondrocytes using: high density monolayer culture(13); pellet culture(131); alginate bead encapsulation(5); agarose gels(18); bioreactors(4); cytokines(132-134) and most recently hypoxia(16). Although conflicting reports exist in the literature(8,135), it is generally accepted that some elements of the chondrocyte phenotype can either be preserved or promoted through the use of such techniques. While initial studies with 3D growth environments focused on chondrocyte morphology, more recent work has shown that despite preservation of the characteristic round cell shape, current 3D techniques yield patterns of ECM gene expression that differ significantly from those observed in native chondrocytes(136-138).

1.3.5.9.1 Hypoxic Cell Culture

Although the chondrogenic effect of hypoxia on monolayer cultured chondrocytes was initially reported over 35 years ago by Marcus(139), hypoxic cell culture conditions were not widely used until very recently. In the past 5 years, reports have emerged that have demonstrated the chondrogenic benefits of hypoxia in such diverse culture systems as: alginate bead culture(15,16,140); micromass culture(138); the hanging drop culture method(141) and Hyalograf culture(142). Murphy and Polak demonstrated that 5% O_2 yielded significantly higher levels of gene expression for aggrecan and collagen II chondrocytes maintained in alginate beads(140). Coyle *et al.* also examined the combined effects of alginate-embedded chondrocytes (AEC) and hypoxic culture. and found elevated gene expression for both aggrecan and collagen II (15). Using the hanging drop method, Martinez *et al*. showed that chondrocytes grown under hypoxic conditions yielded significantly higher levels of gene expression for aggrecan and

collagen II expression while simultaneously showing a reduction in collagen I expression(141). The benefits of hypoxia; however, are not limited to gene expression, because Domm *et al*. have demonstrated that hypoxic growth conditions yield significantly higher levels of collagen II protein in both primary chondrocytes and redifferentiatiated chondrocytes when compared to normoxic growth conditions(16). Finally, the benefits of hypoxia are not limited to chondrocytes, because Robins *et al*. found an increase in aggrecan and collagen II gene expression in mesenchymal stem cells grown under hypoxic conditions(74). Based on these studies hypoxia is clearly an important physiological factor that should not be overlooked; however, even in conjunction with some of the most innovative 3D culture techniques it has not yet be proven to either completely retain or completely restore the chondrocytic phenotype, thus necessitating further research.

1.3.5.10 Treatment of Chondral Defects

In addition to disease related alterations seen in OA, damage to AC can also arise through localized trauma. Currently there are a wide range of options available for the treatment of chondral defects ranging from the non invasive all the way up to the replacement of the entire joint with a prosthetic device. Depending upon a variety of factors including age, weight, activity level as well as type and extent of the chondral defect, something as simple as a life style change my be indicated. In situations where cartilage surrounding the primary defect appears abnormal, it may be necessary to perform a technique known as debridement(143). Debridement consists of surgically removing any abnormal or unstable pieces of cartilage surrounding the defect that may be impacting joint function and/or contributing to pain. In patients without osteoarthritis this simple procedure, when combined with abrasion of the subchondral bone, has been shown to improve symptoms for in excess of 5 years(144). In patients with OA, however, the results for the use of this procedure are conflicting and many argue that debridement is not indicated.

1.3.5.10.1 Microfracture and Drilling

Two other techniques known as microfracture and drilling have been performed for over 20 years on larger and/or deeper chondral defects(143). While the instruments used in the respective techniques differ, the objective remains the same. The goal is to access stem cells within the bone marrow by creating numerous tiny perforations in the subchondral bone which will ultimately lead to the formation of a superclot. Over time this clot is replaced but in only about 11% of the cases will it be replaced with a material resembling hyaline cartilage(145). It is therefore not surprising that this technique does not produce clinically acceptable results beyond about 5 years(146).

1.3.5.10.2 Mosaicplasty

Mosaicplasty is a technique that was pioneered about a decade ago and involves the transplantation of osteochondral cylinders to fill a large chondral defect. Usually multiple osteochondral dowels are taken for transplantation from the periphery of the femoral condyles and positioned within the defect thus creating a mosaic appearance. This technique can be performed arthroscopically, but due to its complexity, it is most often performed as an open procedure(143). Although mosaicplasty has the advantage of being able to fill the defect immediately with mature hyaline cartilage, there are numerous variables to consider, thus making it a very complex technique. Despite its complexity, good to excellent results were obtained in over 85% of the cases in a study that was conducted for up to 10 years(147).

1.3.5.10.3 Total Knee Arthroplasty

Currently the “gold standard” treatment for advanced OA of the knee is total knee replacement surgery also known as total knee arthroplasty (TKA). There are almost 400 000 TKAs performed each year in the US alone and that number is expected to almost double over the next two decades(148). This procedure consists of replacing the distal end of femur and the proximal end of the tibia with a prosthetic implant which is designed to restore painless articulation in the joint

albeit with a limited range of movement. With recent advances in materials and techniques the survivorship of the implants has risen to 94-98% at 10 - 14 years(148). Despite these encouraging numbers, prosthetic failure remains very predictable and almost exclusively results from polyethylene wear and/or component loosening(146).

1.3.5.10.4 Autologous Chondrocyte Implantation

In recent years a new technique has emerged which is known as autologous chondrocyte implantation (ACI) and it holds tremendous potential for the treatment of acute chondral defects. In summary, some of the patient's own chondrocytes are harvested arthroscopically, expanded in 2D tissue culture and subsequently re-injected beneath a periosteal graft. According to Britberg *et al.*, good to excellent initial results were obtained at the 3 year point in 70 % of cases(1). While these numbers appear promising, it is clear from other techniques involving the *de novo* synthesis of hyaline-like cartilage that the real hurdles are the 5 and 10 year post surgery figures. Interestingly, a follow-up study published in 2002, where 2 mm biopsies were taken, has shown that 67% of patients had developed "hyaline-like" cartilage by a mean time of just under 5 years(149). Although further analysis and longer term studies are needed, this could well become the "gold standard" treatment for the majority of acute chondral defects, if indeed true hyaline cartilage is being generated.

1.3.5.10.5 Problems with ACI Repair

To effectively assess and compare these studies a clear set of scoring criteria for the development of hyaline cartilage needed to be employed. While such a system has recently been developed, the original reports of hyaline cartilage were based on qualitative visual assessment techniques that were very subjective rather than more reliable quantitative molecular techniques. This has led to reports for the synthesis of hyaline cartilage up to 24 months following ACI that vary from 10% to 73%(3,21,150). The concern is that hyaline cartilage is likely an essential component in successful long term cartilage repair(151). Results from some

studies like the one from Whittaker *et al.* are reporting that even at 24 months "graft sites showed mostly fibrocartilage with only some hyaline cartilage"(2). Interestingly, studies of chondrocytes grown in 2D culture have shown that they dedifferentiate with increasing passage number into a fibroblast-like cell type(20). Furthermore, these dedifferentiated cells have been found to express collagen I (a major component in fibrocartilage) rather than collagen II which is more characteristic of fibroblasts than mature chondrocytes(152). It is therefore conceivable that the cells that have yielded this *in vivo* fibrocartilage are not mature chondrocytes but dedifferentiated fibroblastic relatives that were produced during the initial cell expansion.

1.3.5.11 Future Research

Before we can really proceed with confidence into the exciting new field of tissue engineering there are many aspects of the basic chondrocyte biology that we need to address. The clinical trials thus far involving ACI have underscored how little we understand about the factors influencing even something as trivial as chondrocyte differentiation under 2D vs 3D conditions. To evaluate these variables we need to develop 3D model systems that very closely simulate the *in vivo* environment. Before we can even begin to develop such a system however we need to identify what the critical variables are that can have an impact in such a 3D model.

1.3.6 References

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CHAPTER TWO: Characterization of Normal Human Articular Cartilage.

2.1 Introduction

The primary function of articular cartilage (AC) is to provide a smooth surface for the articulation of the long bones. The remarkable ability of this avascular tissue ultimately arises from its unique structure and composition. Unlike other tissue types such as heart, kidney and liver which are comprised primarily of cells, articular cartilage is composed mainly of extracellular matrix (ECM). Although the ECM is predominantly comprised of collagen II and aggrecan there is also a long list of minor constituents including; collagens VI, IX, XI, decorin, biglycan, COMP and link protein (1). Chondrocytes, the sole cell type in AC, typically comprise less than 5% of the tissue (2). These sparsely distributed cells are responsible for maintaining and remodeling the comparatively large amount of structurally complex ECM that encases them. Chondrocytes unable to accomplish this task will yield cartilage lacking the appropriate stiffness and resilience to function properly. Even minor alterations in the structure or composition of AC that affect tissue function may ultimately lead to deterioration and disease.

2.1.1 ECM Turnover Rates

Considerable variability exists in the intrinsic rates of turnover between the two main constituents in AC. Collagen type II is a remarkably stable molecule with a half-life of over 100 years (3,4). In contrast, aggrecan has a half-life ranging from days to weeks (5) and is therefore turned over far more frequently. Given the significantly higher renewal rate of aggrecan compared to collagen type II, changes occurring at the biochemical level will readily become apparent. The differences in turnover rates between these molecules mean that aggrecan essentially serves as the canary in the coal mine for both age and disease-related changes occurring within AC.

2.1.2 Age-Related Changes

Given that other tissues in the body show signs of age-associated decline it is not surprising to find similar changes in AC. Bayliss *et al*. (6) demonstrated that there

was a significant reduction in the rate of aggrecan incorporation in AC with increasing age. Due to the critical role that negatively charged GAGs play in the retention of water within the cartilage matrix, even relatively insignificant changes in the structure or distribution of aggrecan can have profound consequences on the mechanical properties of the AC. Interestingly, it is within some of these essential water-retaining molecules that researchers have found some of the most pronounced age-related changes. For example, Plaas *et al*. (7) discovered that there were significant age-associated differences in the length of the chondroitin sulfate chains. In addition to the changes in the length of the GAG chains, there have also been several reports of age-related changes in the relative proportions of GAGs (8-10). Beyond the numerous reports of age-associated changes in aggrecan, there have also been reports of an age-associated decline in AC cellularity. For example, Temple *et al*. in a study of 31 cadaveric AC donors found that cellularity was significant higher in young donors than middle or old age donors (11). While these observations suggest that changes in cellularity and cell function occur with advancing age, it is not clear whether these changes irreversible.

2.1.3 Disease-Related Changes

Although there are currently over 100 different medical conditions that fall under the category of arthritis, the most common is osteoarthritis (OA). OA is a degenerative joint disease characterized by the deterioration of AC and the subsequent erosion of subchondral bone, ultimately resulting in joint pain and dysfunction. (12) A loss of aggrecan from the superficial-most layer of the AC (1,13,14), and a reduction in tissue cellularity (15,16) are among the earliest changes detectable in OA cartilage. Given that similar changes are also seen in normal AC from aging donors, the distinction between age-related and disease-related change is unclear. While the precise cause of OA remains elusive, age represents the largest risk factor for the development of the disease and hence plays a very important yet unidentified role (12). For this reason, further research is necessary into normal AC and early OA cartilage to ascertain if the loss of

aggrecan and reduction in tissue cellularity are distinct factors or part of a disease-related continuum.

To gain insight into the range and extent of cellular, histological and immunohistochemical variability present in normal AC, ten normal cartilage samples were analyzed. The range in variability established for normal AC in this descriptive study will be used for comparison purposes during the subsequent analysis of OA-derived tissue.

2.2 Materials and Methods

Normal articular cartilage (AC) from ten femoral condyles was obtained from six human cadavers (Table 2.1) through the Comprehensive Tissue Center and the Human Organ and Procurement Exchange (HOPE) program at the University of Alberta Hospital. From each of the 10 AC samples, a single one centimeter diameter cylindrical core of AC and underlying bone (dowels) was harvested from the central load-bearing region using a stainless steel coring tool. Although all ten normal AC dowels were free of any macroscopic signs of OA, based on macroscopic inspection, three patients did display some minor, localized surface irregularities in other portions of the joint. The normal AC donors ranged in age from 19 to 54 years (42.5 +/- 12.2 yrs).

All dowels were washed in PBS whereby serial 70 μm thick sections, were obtained from the midsagittal region of each dowel using a vibratome. Three of these sections/dowel were immediately stained for cell viability. A scalpel was used to separate the remaining cartilage from the underlying bone at the cartilage-bone junction and the remaining AC was fixed in 4% formalin.

2.2.1 Viability Staining

Three 70 μm sections from each dowel were incubated in Live-Dead reagent (Invitrogen, Cat # L3224) for 30 min according to the manufacturer's recommendations. The sections were washed in PBS and visualized on a Leica

DM/RE fluorescence microscope using either an Alexa 488 filter to reveal the live cells (green) or an Alexa 555 filter to reveal the dead cells (red). By adjusting the depth of field to the middle of the 70 μm tissue section it was possible to count all of the cells in a given section and hence permit cell density calculations. Photomicrographs of representative regions within the sections were taken using a CoolSnap CCD camera. A standardized sampling box of known area was digitally added within the transitional layer of each of the images and the number of live and dead cells within the box was counted. From these data, cell viability and total cell density were calculated for each sample (Table 2.2). The arrangement of chondrocytes in relation to each other (eg. small clusters of up to 3 cells vs larger clusters of 4 or more cells) was also noted.

2.2.2 Histology

Formalin-fixed AC samples were embedded in paraffin, sectioned at 5 μm intervals perpendicular to the articular surface and mounted on Superfrost Plus glass slides. Immediately prior to staining, sections were deparaffinized using ProPar (Anatech Ltd) and rehydrated in a graded series of ethanol washes. Deparaffinized sections were subjected to routine H&E staining to show overall tissue morphology and safranin-O staining to reveal the distribution of proteoglycans within the tissue. Safranin-O staining was achieved by incubating the sections in a 5% safranin-O solution for 5 min, followed by a 20 second wash in water. All sections were dehydrated through a series of graded ethanol washes for 20 seconds each, mounted and imaged at 50x magnification.

2.2.3 Histologic Scoring

Micrographs of the H&E and safranin-O stained AC sections were graded using the Mankin Scoring System (13).

2.2.4 Immunohistochemistry

Formalin-fixed 5 μm sections were deparaffinized and subjected to an antigen retrieval step consisting of a 60 min digestion in 5 mg/ml pepsin, pH 2.0 (Sigma

Cat # P-7000). Sections were rinsed thoroughly in water to remove the pepsin and endogenous peroxidase activity was quenched using a 30 min incubation in 3% H_2O_2 in methanol. Immunohistochemistry was performed using Vectastain Elite ABC kits (Vector Laboratories, Cat. # 6102 and 6105) according to the manufacturer's directions. Primary antibodies [mouse anti-collagen type I (MAB 3391, Chemicon) or mouse anti-collagen type II (MAB 8887, Chemicon) or Rabbit anti-aggrecan (AF-1220, R&D Systems] were applied at a 1:100 dilution in PBS to separate sections from each sample and incubated for 30 min in a humidified chamber. Sections were washed in PBS and biotinylated secondary antibodies were applied for 30 min followed by washing in PBS and incubation for 30 min in VectaStain ABC reagent. Sections were subsequently washed in PBS and exposed to metal-enhanced DAB (Pierce Cat # 1856090) for 5 min The reaction was stopped by rinsing the sections thoroughly in water. Stained tissue sections were dehydrated using a series of graded ethanol washes, mounted and imaged. Sections of human anterior cruciate ligament (positive control for collagen type I and negative control for collagen type II) were included as controls in each series of staining. Sections in which primary antibodies were omitted were also included to determine levels of any non-specific staining.

2.3 Results

2.3.1 Physical Properties

Macroscopically, the semi-translucent, white AC appeared smooth (Fig. 2.1) however; minor microscopic structural disruptions in the superficial layer were observed in 30% of the normal samples (Fig 2.2a-c). Mankin score within the normal samples ranged from 0 to 3 with a mean score of 1.8 with a standard deviation of 0.8. The mean AC depth of the ten samples, not including the calcified cartilage layer, was 1.73 cm with a standard deviation of 0.18 cm.

2.3.2 Cellularity

The highest cell densities were observed in the superficial layer of normal AC while the deep layer typically displayed the lowest cell densities (Fig 2.3a-b). Cell

density did not appear to change with depth throughout the transitional layer. The mean cell density in normal AC was 10,892/mm^3 (Table 2.2). Mean values for numbers of live, dead, total, percent viable and cell density for normal tissue are presented in Table 2.2. Viability in normal AC only attained a mean value of 85.7 which is considerably lower than the expected theoretical value (100%) (Table 2.2). Although dead cells were observed in all normal AC layers, a disproportionate amount of the dead cells resided in the superficial layer. Chondrocyte clusters ranging in size up to 10 cells were observed in the lower transitional and deep layers of normal AC (Fig 2.3a).

2.3.3 Histological and IHC Staining

Aside from minor irregularities in the superficial-most layer, H&E stained sections of normal AC displayed characteristic structure and organization (Fig. 2.4a). A reduction in safranin-O staining was observed in the majority of the normal AC samples (Fig. 2.4b). Pericellular and territorial regions throughout the transitional layer stained less intensely for safranin-O than the surrounding inter-territorial regions. Immunohistochemical staining for aggrecan revealed a similar reduction in staining in the superficial layer. In general aggrecan staining was quite homogeneous throughout the transitional layer of normal AC however; the pericellular regions did not stain as intensely as the rest of this layer. (Fig. 2.4e). In contrast to the transitional layer, the deep layer of normal AC displayed predominantly pericellular staining for aggrecan with only low levels of staining observed in the inter territorial spaces.

In the superficial layer of normal AC, collagen type II had a slightly more pericellular distribution than the remainder of the AC. Collagen type II staining displayed an evenly mottled appearance throughout the transitional layer. Staining in the deep layer was the least intense and did not display a mottled appearance like the transitional layer. (Fig. 2.4c)

Trace amounts of collagen type I staining were either detected at the surface or within the superficial layer of 9 out of 10 normal AC samples (Fig. 2.4g). Control AC sections were devoid of staining when primary antibodies to the respective ECM molecules were omitted (Fig. 2.4d,f,h). Sections of human anterior cruciate ligament, which were employed as a positive collagen type I, yielded intense staining throughout (data not shown).

2.4 Discussion

The samples in this study displayed many of the expected physical properties for normal human AC. The range in Mankin score observed in these samples was also consistent with the range of tissue damage reported in normal AC by other studies. Cell densities in the normal AC samples were also consistent with the average value of 9 626/ mm^3 previously reported by Hunziker *et al*. (17). Although a study by Temple *et al*.,(11) reported an age-related decline in cellularity, in the current study, when the mean cell density was compared to published values, there was no evidence of a decline in cellularity. Despite an absence of change in cell density, changes in cellularity such as chondrocyte clustering were observed (Fig 2.2a.). Interestingly, the clustering observed here was similar to a recent report by Bentley *et al.* (18) in which they found that chondrocyte clustering was one of the earliest events in the progression of OA. It should be noted that the mean viability observed in normal AC (85.7%) was considerably lower than expected. Because reduced viability remained relatively constant across the samples, it was likely an artifact of either the tissue harvesting (drying-related) or tissue sectioning process (mechanical disruption) rather than an *in vivo* cell death.

During OA progression, aggrecan which is the most labile component in the ECM typically shows signs of loss in the superficial layer well in advance of other disruptions. While this is attributed to the exposure of the tissue to the proteolytic enzymes within the knees of OA patients [reviewed in (19,20)], the loss of aggrecan from the superficial-most layer was also observed in the normal AC

samples. Since this tissue was obtained from otherwise normal patients, it suggests that the loss of aggrecan may represent a continuum which spans the boundary between normal AC and early OA. In normal AC, the marginal loss of aggrecan from the superficial may result from reversible wear and tear-related the remodeling, whereas the advanced loss of aggrecan in OA may represent a loss of regulation of the normal remodeling process.

Much controversy exists in the literature regarding the expression of collagen type I in AC. Some studies in OA have failed to detect collagen type I (21,22) while others have reported that it is restricted to AC in the late stages OA (23-25). In contrast to earlier reports, a recent study found that collagen type I can indeed be present in the superficial layer of normal AC(26). The current study supports the latter finding, however; the mean age of the patients this study, which borders on the age of onset for OA, represents a potential confounding variable.

The appearance of minor structural irregularities in load bearing regions of normal AC seems to be a fairly common occurrence and may simply reflect the damage from normal wear and tear. Fernandes *et al*. reported that many otherwise healthy joints show signs of roughness and minor fibrillation which supported our observation(27). Although collagen type I was recently found to be associated with structural damage in OA, faint staining was nonetheless detected in the superficial layer of normal AC. Given the age of the patients in the study, this could be indicative of impending structural tissue failure; however it may simply represent a mechanism for the repair of the minor structural irregularities that were observed.

2.4.1 Summary

When taken together, the prevalence of microscopic irregularities in conjunction with the loss of aggrecan and presence of collagen type I in the superficial layer appears to suggest that normal AC has the capacity to initiate a tissue repair response in response to minor wear and tear–related damage.

Figures

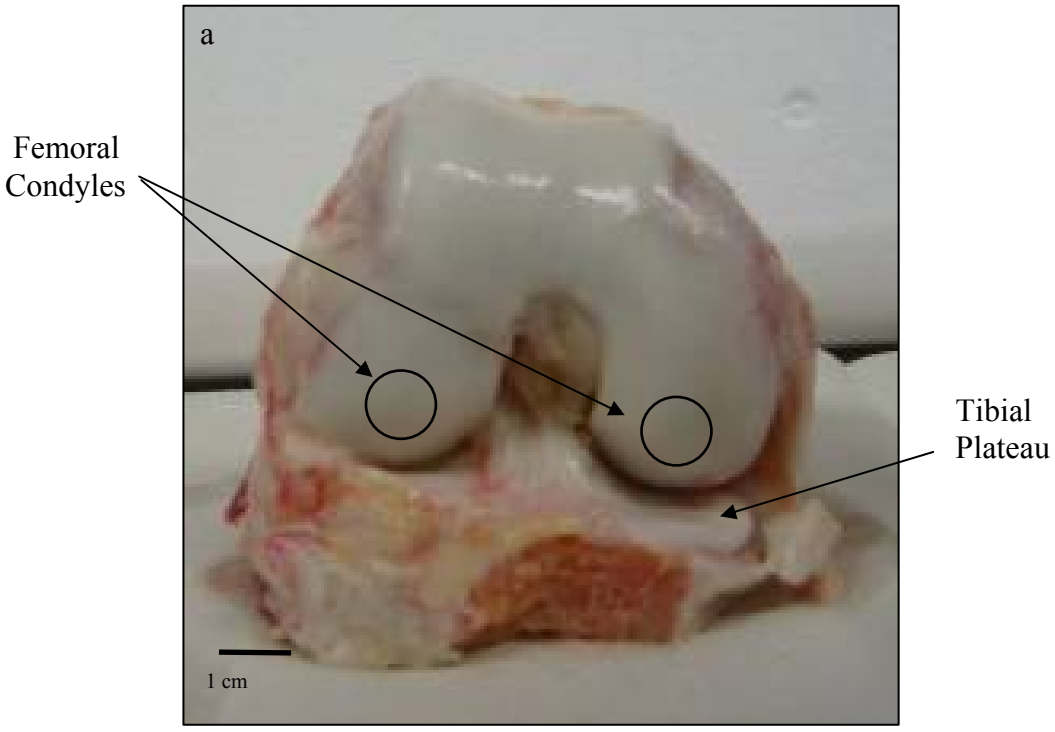

Figure 2.1. Image showing typical: smooth, white, OA-free tissue obtained from normal AC donors. Circles represent regions of 1 cm diameter within load bearing regions from which dowels were collected. Bar = 1 cm.

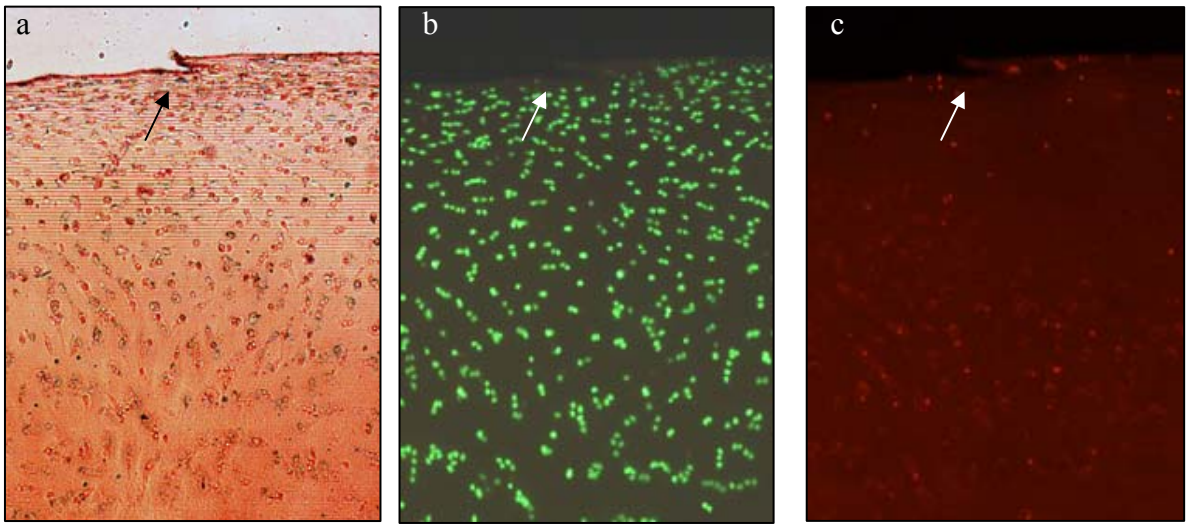

Figure 2.2. Micrographs of Live-Dead stained cells showing a typical structural irregularity in the superficial layer of a typical 70 µm section of AC. Images showing a) overall tissue morphology b) live cells c) dead cells. Bar = 50 µm.

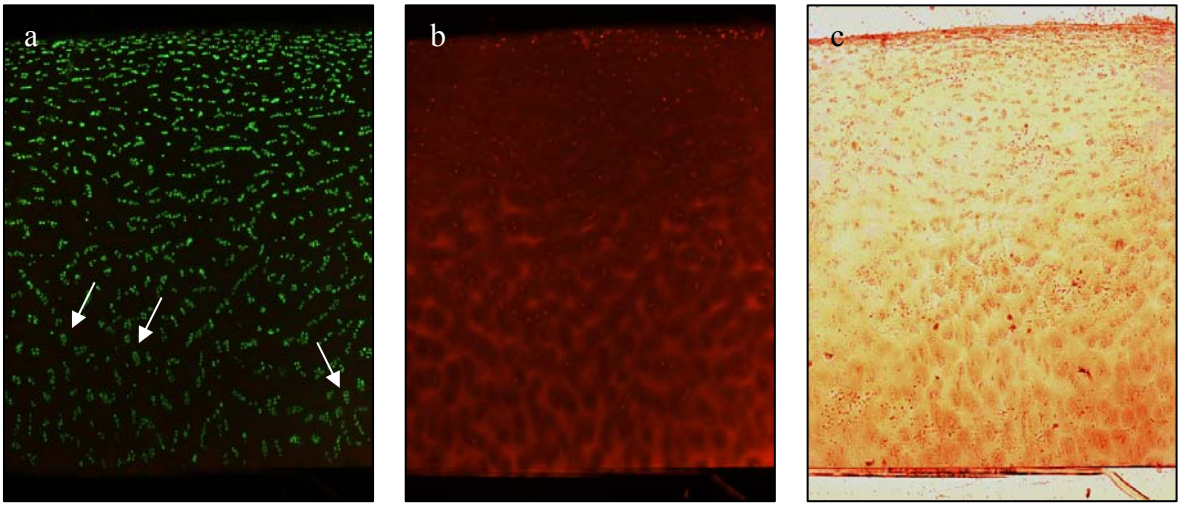

Figure 2.3. Micrographs of Live-Dead stained cells showing the distribution of chondrocytes in a typical 70 µm section of AC. Images showing a) live cells b) dead cells c) overall tissue morphology. Arrows indicate cell clusters. Bar = 100 µm.

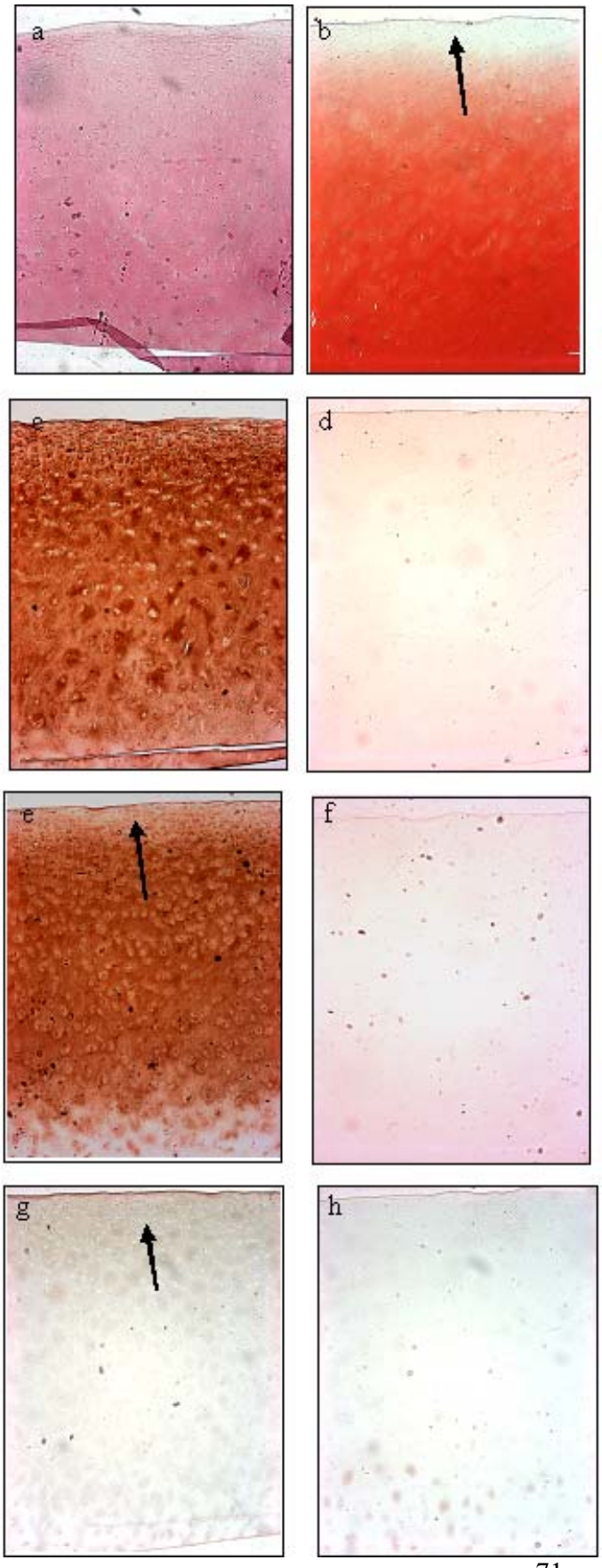

Figure 2.4. Micrographs showing normal articular cartilage stained with a) H&E, b) Safranin-O c) collagen type II d) collagen type II (no primary ab) e) aggrecan f) aggrecan (no primary ab) g) collagen type I h) collagen type I (no primary ab). Arrows indicate regions showing a loss of respective ECM component. Arrows indicate alterations in staining for aggrecan and collagen type I. Bar = 100 μm.

Tables:

Table 2.1. Sample information including age sex and patient mass of patients.

Name	Age	Sex	Mass
Hac6	54	Male	75 kg
Hac8	48	Female	58 kg
Hac9	44	Male	75 kg
Hac10	19	Female	55 kg
Hac16	48	Female	61 kg
Hac17	42	Male	77 kg

Table 2.2. Mean numbers (+/- SD) of live, dead and total cells for normal AC. Percent viability and cell density are also presented.

Grade	Live	Dead	Total	Viability(%)	Cells/mm^3
Norm	311.0 (63.1)	51.1 (33.0)	362.1 (61.2)	85.7 (9.4)	10892

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CHAPTER THREE: Collagen I Deposition in Early OA and Associated Changes in Articular Cartilage Metabolism.*

3.1 Introduction

The primary function of articular cartilage (AC) is to provide a smooth surface for the articulation of the long bones. The remarkable ability of this avascular tissue ultimately arises from its unique structure and composition. AC is composed primarily of extracellular matrix (ECM), with chondrocytes accounting for less than 5% of the tissue (1). Although the ECM is predominantly comprised of collagen type II and the proteoglycan aggrecan, there is a long list of minor constituents including; collagen type VI, collagen type IX, collagen type XI, decorin, biglycan, hyaluronan, cartilage oligomeric protein and link protein [reviewed in (2)]. The arrangement and distribution of these constituents within the AC are responsible for endowing it with its unique mechanical properties and tensile strength (3). Due to the interdependence of the various constituents, even minor alterations may lead to a series of events culminating in the loss of tissue function and even disease.

3.1.1 Primary Alterations in OA Tissue

Osteoarthritis (OA) is a degenerative joint disease characterized by pain and dysfunction which represents a major cause of disability worldwide (4). Although the cause of primary human OA remains unknown, the disease is characterized by a progressive breakdown of the ECM. In OA it has been shown that proteoglycans residing in the superficial layer of human AC are the first components of the ECM to be affected (5,6). Given that similar changes in proteoglycans have also been observed in aging AC (7-11), the extent of the role played by them in the initiation of human OA and subsequent early disease progression remains unclear. An alternative explanation for the onset of OA suggests that changes in proteoglycans merely serve as a prerequisite for the initiation of disease. It has been proposed that an initial loss of aggrecan may allow matrix metalloproteinases access to the collagen framework (12) whereby they can cause denaturation and subsequent loss of collagen type II from the surrounding area

(13,14). Because collagen type II has an exceedingly low turnover rate, with an expected half-life in excess of 100 years (15,16), the loss of this otherwise stable component may represent the first irreversible step in the disease process. Further research into the earliest stages of human AC deterioration is necessary to clarify the relationship between collagen type II, aggrecan and other molecules (such as collagen I) during the onset and subsequent progression of OA.

3.1.2 Alterations in AC Metabolism

Prior to the discovery that chondrocytes in degenerative canine AC displayed upregulated collagen synthesis (17), OA was generally viewed as the result of a series of catabolic processes. While the initial stage of human OA is still primarily characterized by ECM catabolism (18-21), subsequent stages of OA are now known to exhibit an array of anabolic processes (20,22-27). A recent study by Lorenzo *et al.* reported differences in tissue metabolism between specimens representing early and late OA. They found that early stage OA specimens displayed only low levels of collagen type II synthesis, while late stage specimens displayed very high levels (20). This observation provided evidence for the existence of a switch in tissue metabolism during human OA progression. To better understand the nature of this metabolic transition and the factors involved in it, a continuum of human OA specimens ranging from macroscopically normal to moderately fibrillated needs to be analyzed at the molecular level.

3.1.3 Collagen I in AC

In addition to disease-related changes in the expression of aggrecan and collagen type II, there are also reports of the aberrant expression of collagen type I in OA. Although one study has suggested that collagen type I is present on the surface of normal human AC (28), it is generally accepted that collagen type I does not form part of the ECM in healthy AC. In contrast, more controversy has existed over the expression of collagen type I in human OA tissue. While some studies have found collagen type I in the AC of patients with advanced OA (24) or present in the fibrocartilage associated with advanced OA (25), other studies have found no

evidence of collagen type I expression in the AC of OA patients (27,29). Using a rabbit model, Pfander *et al*. (30) reported that collagen type I was deposited in the superficial, fibrillated region of OA cartilage. However, it remains unclear whether deterioration resulting from induced joint instability in this animal model sufficiently approximates the collagen type I expression that occurs in primary human OA. For this reason a range of human OA specimens with and without fibrillation should be examined for the presence of collagen type I staining to confirm these findings. Since reports of collagen I in OA have either been sporadic or anecdotal, a more comprehensive study is required to determine 1) the routine presence of collagen I in human OA tissue 2) its location within the AC 3) time of initial appearance and presence in subsequent stages and 4) what molecular/structural changes are associated with its appearance.

To gain further insight into the role played by collagen I in OA progression as well as the factors responsible for the subsequent change in chondrocyte metabolism during human AC deterioration, this study employed immunohistochemical (IHC) techniques to examine thirty OA samples obtained at arthroplasty that exhibited a range of degenerative changes. These samples were assessed for deposition of collagen type I, collagen type II and aggrecan. We hypothesized that collagen I is present in much earlier stages of human OA AC than has previously been reported and that it may be part of a natural tissue healing response in AC which could be associated with the previously described increase in collagen II expression. Furthermore, a better understanding of early events in AC deterioration may yield new avenues for therapeutic intervention that could halt OA progression in the early stages of disease, thus avoiding the need for joint replacement.

3.2 Materials and Methods

3.2.1 Tissue Collection and Processing

The Outerbridge (OB) grading system (31), which is widely used for the macroscopic assessment of OA cartilage during surgery, was employed to ensure

that cartilage samples representing a clinical range of early tissue degeneration were collected. OA tissue from femoral condyles was obtained during surgery for total knee arthroplasty (TKA) (Fig. 3.1a). The tissue was immediately placed in saline solution and stored at 4°C for up to 24 hrs. One centimeter diameter cylindrical cores of AC and underlying bone (dowels) were obtained from load-bearing regions using a custom-made stainless steel coring tool. Dowels representing OB grades 0 (normal looking AC), I (minor AC irregularities) and II (moderate AC fibrillation) were collected for analysis. A total of 30 dowels (ten dowels from each OB grade) were collected from 17 OA patients (7 males and 10 females) who ranged in age from 58 to 84 years (67.4 +/- 7.4 years). Normal AC from femoral condyles was obtained from six fresh human cadavers through the Comprehensive Tissue Center and the Human Organ Procurement and Exchange (HOPE) program at the University of Alberta Hospital (Fig. 3.1b). These patients ranged from 19 to 54 years of age (42.5 +/- 12.2 yrs). Ten normal AC dowels that were free of any macroscopic signs of OA were harvested from these six cadaveric donors. Three of the cadaveric joints displayed minor, localized surface irregularities; therefore, all dowels were harvested one centimeter or more away from these areas. A scalpel was used to separate the cartilage from the underlying bone at the cartilage-bone junction and the AC was fixed in 4% formalin.

3.2.2 Histology

Formalin-fixed AC samples were embedded in paraffin, sectioned at 5 μm intervals and mounted on Superfrost Plus glass slides (#12-550-15 Fisher Scientific, Ottawa, ON). Immediately prior to staining, sections were deparaffinized using ProPar (Anatech Ltd, Battle Creek, MI) and rehydrated in a graded series of ethanol washes. Deparaffinized sections were subjected to routine hematoxylin and eosin (H&E) staining to show overall tissue morphology and safranin-O staining to reveal the distribution of proteoglycans within the tissue. Safranin-O staining was achieved by incubating the sections in a 5% safranin-O solution for 5 min, followed by a 20 second wash in water. All sections were dehydrated through a series of graded ethanol washes for 20 seconds each,

mounted and visualized at 50x magnification on a Leica DM/RE microscope. Photomicrographs of representative regions within the sections were taken using a CoolSnap CCD camera (Fig. 3.2a, b).

3.2.3 Histologic Scoring

Micrographs of the H&E (Fig. 3.3a-f) and safranin-O (Fig. 3.3g-l) stained AC sections were visually evaluated and arranged based on their level of tissue deterioration according to the Mankin Scoring System (6).

3.2.4 Immunohistochemistry

Formalin-fixed 5 μm sections were deparaffinized and subjected to an antigen retrieval step consisting of a 60 min digestion in 5 mg/ml pepsin, pH 2.0 (P-7000 Sigma-Aldrich, Oakville, ON). Endogenous peroxidase activity was quenched using a 30 min incubation in 3% H_2O_2 in methanol. All immunohistochemistry was performed using Vectastain Elite ABC kits (# 6102 and 6105, Vector Laboratories, Burlingame, CA) according to the manufacturer's directions. Primary monoclonal antibodies [mouse anti-collagen type I (MAB 3391, Millipore, Billerica, MA) or mouse anti-collagen type II (MAB 8887, Millipore, Billerica, MA) or polyclonal rabbit anti-aggrecan (AF-1220, R&D Systems Minneapolis, MN)] were applied to separate sections from each sample. Sections were exposed to metal-enhanced DAB (# 1856090, Thermo Scientific, Rockford, IL) for 5 min. Stained tissue sections were dehydrated using a series of graded ethanol washes, mounted, visualized and imaged as indicated above (Fig. 3.3). Further tissue unmasking with citrate buffer or an equivalent solution was not required with the above-mentioned antibodies. Sections of human anterior cruciate ligament, stained with antibodies raised against collagen type II (Fig. 3.4a) and collagen type I (Fig. 3.4b) were included as negative and positive controls respectively. Sections in which primary antibodies were omitted showed no significant signs of non-specific staining (data not shown).

3.3 Results

3.3.1 Collagen Type I

Collagen type I staining intensity and distribution (Fig. 3.3m-r) displayed a direct association with structural damage and an inverse association with proteoglycan staining (Fig. 3.3g-l). For example, in MG-1 samples, which showed no signs of structural damage (Fig. 3.3b) and only a minor reduction in superficial proteoglycan staining (Fig. 3.3h), there was only a faint trace of collagen type I in the superficial-most layer (Fig. 3.3n). Conversely, MG-2 samples which displayed some minor structural damage in the superficial layer (Fig. 3.3c) showed diffuse collagen type I staining in the upper portion of the section (Fig. 3.3o) and a distinct loss of proteoglycan staining (Fig. 3.3i). MG-3 samples, which showed evidence of structural damage (Fig. 3.3d), typically displayed more intense collagen type I staining (Fig. 3.3p) and displayed a greater loss of proteoglycan staining in the superficial layers (Fig. 3.3j). The association between elevated amounts of structural AC damage and the increased intensity and distribution of collagen type I continued in samples with MG 5-8. AC with damage ranging from major excavations (Fig. 3.3e) to moderate fibrillation (Fig. 3.3f) showed intense collagen type I staining (Fig. 3.3q, r) and an absence of proteoglycan staining throughout much of the upper third of the tissue (Fig. 3.3k, l). Although collagen type I staining was predominantly associated with structural damage in the superficial and upper transitional layers, staining was also observed in the lower transitional and deep layers in a highly localized manner surrounding isolated fissures (data not shown).

3.3.2 Collagen Type II

Major changes were observed in the collagen type II staining pattern during the course of early AC deterioration. In normal samples, collagen type II staining displayed a homogeneous mottled appearance from the superficial through the transitional layer, with less staining observed in the deep layer (Fig. 3.3s). In contrast, the OA samples displayed a wide range of collagen type II staining in both intensity and distribution (Fig. 3.3t-x). For example, MG-1 AC which

typically only showed minor proteoglycan loss (Fig. 3.3h) and possessed an intact lamina splendens (Fig. 3.3b), displayed a loss of collagen type II staining from the superficial layer (Fig. 3.3t) when compared to normal AC (Fig. 3.3s). Sections representative of MG-2 AC showed a more pronounced loss of collagen type II with reduced staining throughout the superficial and transitional layers (Fig. 3.3u). MG-3 AC, displaying structural damage in the superficial layer (Fig. 3.3d) and a loss of proteoglycans into the transitional layer (Fig. 3.3j) showed elevated deposition of collagen type II in the superficial and transitional layers (Fig. 3.3v) compared to MG-2 (Fig. 3.3u). In samples representing intermediate levels of OA progression (MG 5-8), staining for both collagen type I and collagen type II seemed to co-localize throughout the fibrillated superficial region and underlying transitional zone. Collagen type II staining was most intense in fibrillated AC and comparable to the levels of collagen type I. In contrast to collagen type I, intense localization of collagen type II occurred subsequent to the appearance of structural damage rather than preceding it. In the transitional and deep layers of samples displaying overt structural damage (MG 5-8), collagen type II deposition in the territorial regions became increasingly pronounced with disease progression (Fig. 3.3w, x). The immunohistochemical staining yielded discrete rings surrounding the cells which corresponded to the boundary between the territorial and inter-territorial regions (Fig. 3.5a).

3.3.3 Aggrecan

In normal samples, the pattern of aggrecan staining was homogeneous from the superficial through the transitional layer with less intense staining observed in the lower half of the deep layer (Fig. 3.3y). In contrast, all OA samples, even those with a normal macroscopic appearance, exhibited reduced aggrecan staining in the superficial-most layer when compared to normal controls (Fig. 3.3z-dd). In the earliest stages of AC degeneration (MG-1 and MG-2), aggrecan loss from the superficial-most layer (Fig. 3.3z, aa) paralleled the loss of collagen type II (Fig. 3.3t, u). In subsequent stages of progression, a marked difference emerged between the staining patterns for aggrecan and collagen type II. For example,

MG-3 AC displayed a major loss of aggrecan from the superficial and upper transitional layers (Fig. 3.3bb) while collagen type II staining (Fig. 3.3v) showed evidence of elevated deposition in those regions. Unlike collagen type II, aggrecan staining in the superficial layer did not increase in fibrillated specimens (Fig. 3.3cc, dd). Aggrecan staining in MG-3-5 samples (Fig. 3.3cc, dd) was most intense in the interterritorial regions throughout the transitional and deep layers (Fig. 3.5b).

3.4 Discussion

3.4.1 Collagen Type I

Controversy exists in the literature regarding the expression of collagen type I in OA tissue. Some reports fail to detect collagen type I altogether (27,29) while others have found it predominantly associated with late stage disease (24,25,32). In this study, a strong association between the amount of structural damage in the superficial layer and the extent and intensity of collagen type I staining was observed in 28 out of the 30 OA samples. The collagen type I-stained sections showed a progressive increase in the extent of staining with increasing degrees of structural damage such as fibrillation and fissuring. Moreover, collagen type I staining was typically the most intense in regions immediately surrounding the structural damage. Although collagen type I staining was a prominent feature in fibrillated AC (MG 5-8) (Fig. 3.3q, r), it was also clearly detectable in samples representative of earlier OA which displayed only minor superficial disruptions (MG 2-3) (Fig. 3.3o, p). Taken together, the prevalence of collagen type I staining found here and in the study by Pfander *et al.* (30) suggests that collagen type I accumulation may be a common feature of structurally damaged AC. This widespread expression of collagen type I in response to tissue damage appears to be consistent with the natural healing process that occurs in other tissue types (33,34) and therefore need not be attributed to chondrocyte dedifferentiation, as proposed elsewhere (27,30). Interestingly, in a study of 3D growth environments on the maintenance of the chondrocytic phenotype, Takahashi and Takahashi recently provided evidence that suggests a link between cell-matrix interactions

and collagen type I gene expression. They have shown that disruption of cell-matrix interactions, which are mediated via integrins on the cell surface, results in 100 fold increase in collagen type I expression (35). Based on their findings, the disruption of the cell-matrix interactions occurring in OA as the result of the ongoing tissue proteolysis may directly account for the elevated collagen type I seen here and by Pfander *et al*. in structurally damaged AC.

A complex relationship appears to exist between collagen type I and collagen type II during OA progression. In the early stages where minimal macroscopic disruptions in the AC were observed, collagen type II was lost from the superficial layer (Fig. 3.3u) while collagen type I seemed to appear in its place (Fig. 3.3o). Interestingly, the degradation of one collagen type and synthesis of the other, in such close proximity would require that the opposing metabolic processes be tightly controlled, which is in contrast to the classical concept of chaotic disease-related deregulation. In intermediate OA showing signs of macroscopic structural damage, where collagen type II gene expression is typically elevated (32,36), both collagen type I (Fig. 3.3r) and collagen type II (Fig. 3.3x) appeared to be deposited at high levels in the superficial and transitional layers of pre-existing AC. The co-localization of these two collagens may represent an attempt by the tissue to prevent further structural damage that would lead to widespread damage-induced cell death and hence accelerated tissue degeneration. This intense co-localization of collagens type I and II is likely part of the normal repair process in AC which merely precedes the appearance of *de novo* fibrocartilage that was seen during tissue collection for this study and which has been reported elsewhere (24,37).

3.4.2 Collagen Type II

The arrangement and distribution of the various ECM components within AC are responsible for its unique mechanical properties and tensile strength (3). Alterations in any of the major constituents, such as aggrecan or collagen type II, can compromise the delicate equilibrium and contribute to OA progression

(38,39). Based on recent reports, even altered expression of the minor constituents such as collagen type III (27), fibronectin (30) and tenasin (40), may play an important role in disease progression.

In this study, changes in the pattern and intensity of collagen type II immunohistochemical staining during the course of disease were among some of the most striking observations as was expected. Even in the absence of disruptions in the lamina splendens, a loss of collagen type II staining from the superficial-most layer was detected (Fig. 3.3t). In the subsequent stage of disease (MG-2) where minor structural alterations were observed, the loss of collagen type II staining was more pronounced and penetrated deeper into the AC. This observation supports the finding by Hollander *et al*. that collagen type II loss begins at the surface and proceeds deeper with disease progression (13). Interestingly, despite recent gene expression results suggesting that collagen type II anabolism predominates in early OA (41), this study provides evidence that catabolism is the prevailing process during the earliest stages of OA. It is possible that Lorenz *et al*. were documenting gene expression at an intermediate stage of OA and not the earliest stages as investigated in this study. In contrast to the loss of collagen type II staining seen in early OA (ie. MG 1-3) (Fig. 3.3t-v), intense collagen type II staining was observed in the superficial-most layer of tissue representing intermediate stages of OA (MG 5-8) (Fig 3.3w, x), consistent with Lorenz *et al*. For example, regions of AC displaying signs of macroscopic damage such as fibrillation typically displayed the most intense collagen type II staining. This observation in human AC is similar to that of Pfander *et al.* (30) in rabbits. Based on the observed progression of OA, it appears that a threshold to damage tolerance exists between MG 3-5 which marks the boundary between the predominantly catabolic loss of collagen type II in the earliest stages and the anabolic accumulation of collagen type II in the intermediate stages.

3.4.3 Aggrecan

Although there was a progressive loss of aggrecan from the superficial layer with disease progression, the most striking change in aggrecan staining related to its

localization pattern with respect to collagen type II. In the transitional and deep layers of fibrillated OA cartilage (MG 5-8) (Fig. 3.3w, x), collagen type II localized intensely to the territorial regions immediately surrounding cells (Fig. 3.5a), while aggrecan was predominantly deposited in the interterritorial space between cells (Fig. 3.5b). In normal AC, aggrecan and collagen type II co-localized whereas in the intermediate stages of OA they were spatially segregated throughout the transitional and deep layers. While this may simply reflect delays in assembly and transport of newly synthesized ECM components during the anabolic phase, it may further accelerate disease progression by impeding normal cartilage function.

This extensive descriptive histological analysis of the earliest stages of OA using human OA has confirmed and extended many of the collagen type I and collagen type II observations reported by Pfander *et al*. in a rabbit model (30). Furthermore, our study demonstrated that collagen type I deposition was strongly associated with all types of structural damage and may represent a primary damage response in AC. The intense co-localization of collagen I and II in subsequent fibrillated stages of OA suggested that their expression may be part of an ultimately flawed but natural healing process in AC. Indeed, the over-expression of collagen I at the expense of collagen II expression may lead to the development of inferior fibrous repair tissue that has been noted to occur during stages of cartilage degeneration previously reported (24,37). Suppression of collagen I expression in conjunction with the native augmentation of collagen II production could represent a novel therapeutic approach capable of enhancing the intrinsic repair process resulting in the restoration of the normal cartilage architecture. Therapeutic agents that have this desired outcome may be effective in producing more normal AC after the OA process has begun.

This study has provided new insight into the role of collagen I in the molecular progression of OA, but it is only through further study of human tissue that the intricacies surrounding the development and early progression of the disease will

be fully revealed. A closer examination of intrinsic repair in OA, which appears to be the natural result of anabolic processes, could prove invaluable in developing a better understanding of the natural barriers that currently impede attempts at extrinsic repair.

Figures

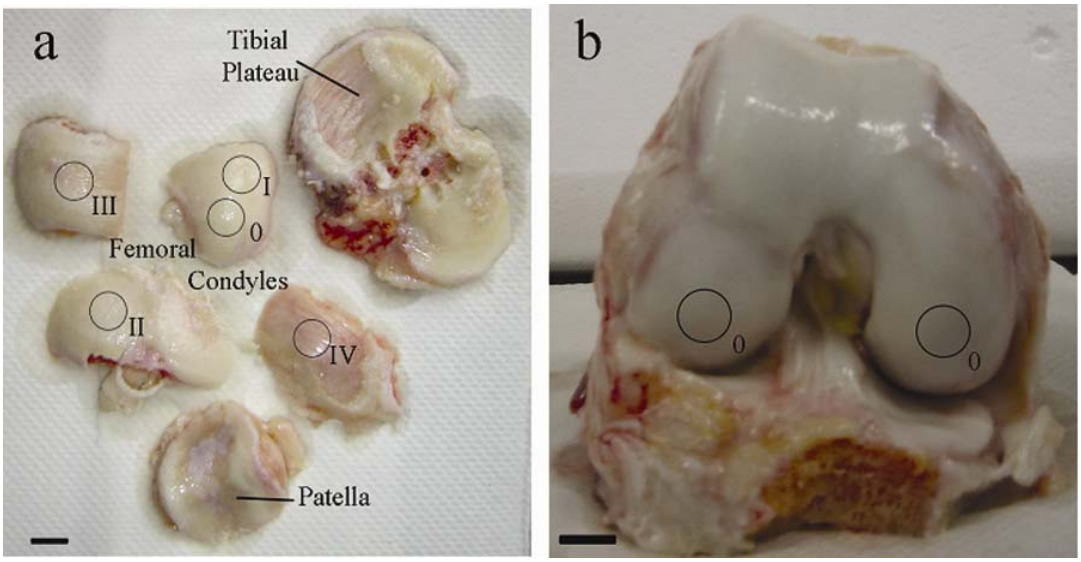

Figure 3.1. Images showing (a) typical total knee arthroplasty fragments from which a wide range of osteoarthritic samples could be obtained and (b) a distal femur from a normal AC donor displaying smooth, white, osteoarthritis-free tissue. For the purpose of this study, Outerbridge scores were assigned to individual osteochondral dowels. The Roman numerals adjacent to circles indicate the Outerbridge grade assigned to each of the dowels. Bar = 1 cm.

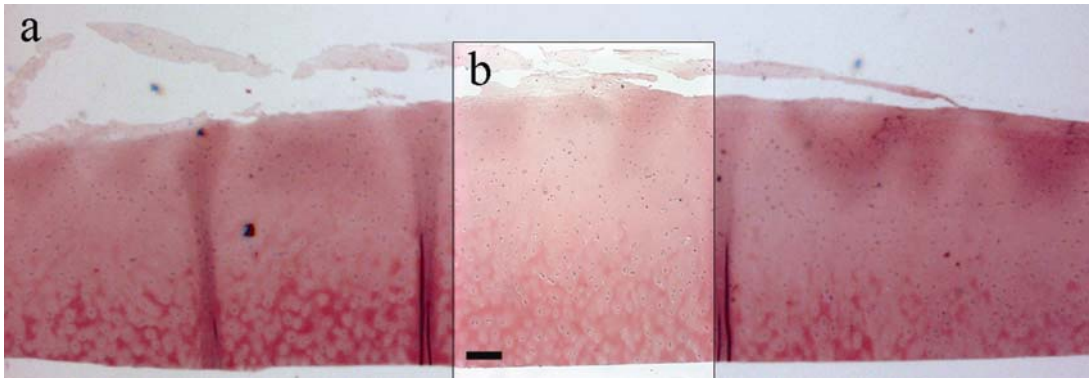

Figure 3.2. Micrograph (a) showing part of a typical AC section stained with H&E and imaged at 16x magnification. The inset (b) shows a representative region within the section that was photographed at 50x for more detailed analysis. Bar = 100 μm.


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Figure 3.3. Representative micrographs of AC stained for H&E (a-f), safranin-O (g-l), collagen type I (m-r), collagen type II (s-x) and aggrecan (y-dd). Cartilage sections are arranged with increasing Mankin score from left to right [Mankin grades 0 (normal), 1, 2, 3, 5 and 8 respectively]. Five distinct steps in the molecular progression of early osteoarthritis are presented. Normal human cartilage is shown on the far left for comparison purposes. Bar = 100 μm.

Figure 3.4. Micrographs showing 5 μm sections of human anterior cruciate ligament stained with antibodies against (a) collagen type II and (b) collagen type I. Note the absence of staining for collagen type II. Bar = 100 μm.

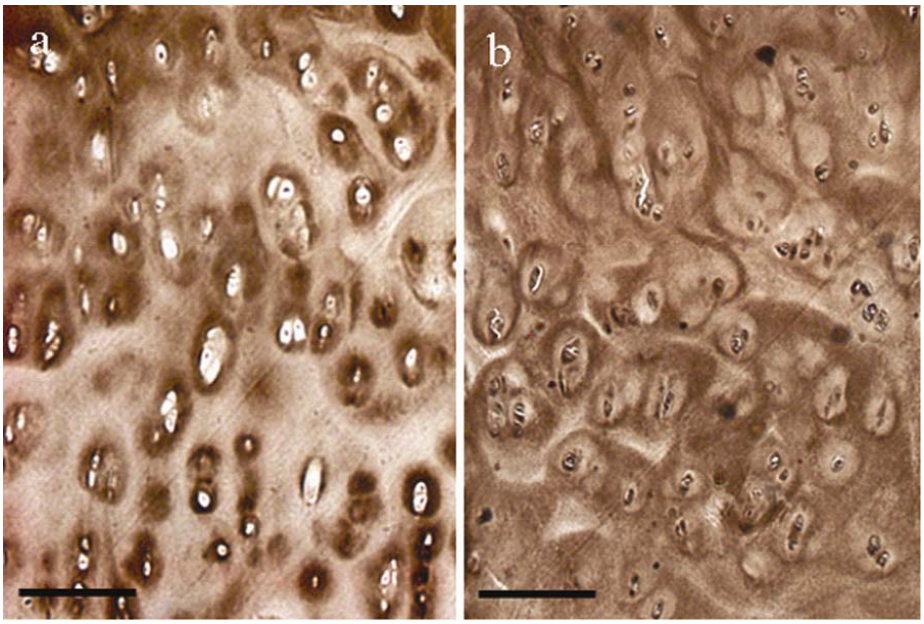

Figure 3.5. Micrographs showing enlarged regions of Fig. 3x and 3dd demonstrating the distribution of collagen type II (a) and aggrecan (b). Note that collagen type II staining was most intense in the territorial region, while aggrecan staining was most intense in the inter-territorial regions. Bar = 100 μm.

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CHAPTER FOUR: Histological Scoring of Articular Cartilage Alone Provides an Incomplete Picture of Osteoarthritic Disease Progression. [#]

4.1 Introduction

4.1.1 Articular Cartilage Scoring Systems

Over the past several decades, a variety of tools for the assessment of articular cartilage (AC) deterioration have been developed (1-6) but, unfortunately, none were designed to assess molecular changes. Macroscopic assessment systems such as Collins (4), Outerbridge (1) and Société Française d'Arthroscopie (SFA) (3), which are relatively rudimentary, continue to be used for arthroscopic assessment, where they provide a convenient yet somewhat inexact (7) method for distinguishing between broad stages of disease progression. Although macroscopic assessment systems are widely used in clinical applications, the limited number of scoring categories that are available in these systems fails to account for many of the important changes occurring at the histological (8), cellular (9-11) and biochemical (12) levels. For this reason, more rigorous scoring systems such as the Mankin Scoring System (5), and Osteoarthritis Research Society International (OARSI) Histopathologic Grading System (6) were developed which permit the distinction of far more subtle changes during AC deterioration.

4.1.2 Histological Scoring Systems

The Mankin Scoring System, also known as the Histological/Histochemical Grading System (HHGS), utilizes a comprehensive 14 point scale for the assessment of AC deterioration (5). However, one criticism of the Mankin Scoring System is that it was developed using advanced osteoarthritic tissue and therefore lacks the ability to adequately distinguish between mild and moderate osteoarthritic (OA) samples (13). The newly developed OARSI System was specifically designed to address this shortcoming and hence, offers better resolution at this level of AC degeneration. Although the OARSI System does appear to offer linearity and greater resolution for early OA, the reliability and validity of this system has not been demonstrated on human AC. Aside from the

above-mentioned deficiencies, the greatest shortcoming with the use of these scoring systems in the future may be that neither one has the ability to account for the molecular changes occurring during disease progression.

4.1.3 Immunohistochemical OA Progression

In the past two decades, great strides have been made towards understanding the immunohistochemistry underlying OA. In AC, the immunohistochemical (IHC) distribution of numerous extracellular matrix (ECM) components has been examined including; collagen type I (14), collagen type II (11), collagen type III (15), collagen type V (16), collagen type VI (17), collagen type IX (18) collagen type X (19), aggrecan (20), tenacin (21), biglycan (22), decorin (23), link (24), COMP (25) and fibronectin (26). Mounting evidence suggests that the presence and distribution of many of these ECM components is altered during disease progression (14,26-29), yet no IHC scoring system or module has been developed to describe and assess these changes. As the molecular understanding of OA progression continues to improve, so should the ability to classify these pathological alterations. Unfortunately, it remains unclear whether IHC analysis will yield information about OA progression beyond what is currently available though conventional histological methods. To determine if additional information about disease progression can be obtained from IHC analysis a range of 30 AC samples spanning Mankin grades (MG) 1-5 were examined for intra-grade discrepancies in collagen type II and aggrecan deposition.

4.2 Methods

4.2.1 Tissue Collection and Processing

OA tissue from femoral condyles was obtained during total knee arthroplasty surgery. Osteochondral tissue was immediately placed in saline solution and stored at 4°C for up to 24 hrs. From all tissue samples, one centimeter diameter cylindrical cores of AC and underlying bone (dowels) were obtained from load-bearing regions (Fig. 4.1) using a stainless steel coring tool. Dowels were collected from 18 OA patients (8 males and 10 females) that ranged in age from

56 to 86 years with a mean age of 68.4 +/- 8.5 years. For the purposes of this study, the cartilage portion of the dowel was considered as the study unit.

4.2.2 Histology

Osteochondral dowels were washed in PBS and the cartilage was removed from the underlying bone at the cartilage-bone junction thus eliminating the need for a lengthy decalcification step that would promote excessive leaching of proteoglycans. The AC was fixed in 4% formalin and embedded in paraffin. Articular cartilage samples were sectioned at a 5 μm thickness and mounted on Superfrost Plus glass slides (#12-550-15 Fisher Scientific, Ottawa, ON). Immediately prior to staining, sections were deparaffinized using ProPar (Anatech Ltd, Battle Creek, MI) and rehydrated in a graded series of ethanol washes. Deparaffinized sections were stained with H&E to show overall tissue morphology and safranin-O staining to reveal the distribution of proteoglycans within the tissue. Safranin-O staining was achieved by incubating the sections in a 5% safranin-O solution for 5 minutes, followed by a 20 second wash in water. All sections were dehydrated through a series of graded ethanol washes for 20 seconds each, mounted and visualized at 50x magnification on a Leica DM/RE microscope. Photomicrographs of representative regions within the sections were taken using a CoolSnap CCD camera.

4.2.3 Histologic Evaluation

Photomicrographs of the H&E and safranin-O stained sections were scored using the modified Mankin Scoring System (Table 4.1). Staining artifacts resulting from creasing or folding of the cartilage sections were not considered for analysis. Six AC samples from Mankin Grades 1-5 were identified resulting in 30 AC samples in the study. The tide mark criterion was omitted from the histological scoring because tissue processing made it impossible to evaluate.

4.2.4 Immunohistochemistry

Formalin-fixed 5 μm thick AC sections were deparaffinized and subjected to an antigen retrieval step consisting of a 60 minute digestion in 5 mg/ml pepsin, pH 2.0 (P-7000 Sigma-Aldrich, Oakville, ON). Sections were rinsed thoroughly in water to remove the pepsin and endogenous peroxidase activity was quenched using a 30 minute incubation in 3% H_2O_2 in methanol. All IHC analysis was performed using Vectastain Elite ABC kits (# 6102 and 6105, Vector Laboratories, Burlingame, CA) according to the manufacturer's directions. Primary antibodies [mouse anti-collagen type II (MAB 8887, Millipore, Billerica, MA) or rabbit anti-aggrecan (MAB 1220, R&D Systems Minneapolis, MN] were applied at a 1:100 dilution in PBS to separate sections from each donor sample and incubated for 30 minutes in a humidified chamber. Sections were washed in PBS and biotinylated secondary antibodies were applied for 30 minutes followed by washing in PBS and incubation for 30 minutes in VectaStain ABC reagent. Sections were subsequently washed in PBS and exposed to metal-enhanced DAB (# 1856090, Thermo Scientific, Rockford, IL) for 5 minutes. The reaction was stopped by rinsing the sections thoroughly in water. Stained tissue sections were dehydrated using a series of graded ethanol washes, mounted and imaged as outlined above. Further tissue unmasking with citrate buffer or an equivalent solution was not required with the above-mentioned antibodies. Sections of human anterior cruciate ligament, stained with antibodies raised against collagen type II, were included as negative controls. Sections in which primary antibodies were omitted showed no significant signs of non-specific staining.

4.3 Results

4.3.1 Mankin Scoring System vs. IHC Staining

The range of IHC differences within AC samples of the same MG increased with increasing amounts of cartilage deterioration. In general, MG-1 samples displayed the narrowest range of IHC staining patterns, while MG-5 displayed the broadest range. As a result, IHC techniques revealed marked differences between AC samples that could not have been predicted on the basis of histologic staining

alone. For example, samples classified as MG-1 displayed differences in the extent of staining for both collagen II (Fig. 4.2e,f) and aggrecan (Fig. 4.2g,h), despite similarities in structural integrity (Fig. 4.2a,b) and safranin-O staining (Fig. 4.2c,d). Sample 298-1 displayed a relatively minor loss of collagen type II and aggrecan staining from the superficial AC layer, while sample 300-1 showed an almost complete absence of both components from the corresponding region. The loss of aggrecan in sample 300-1 also extended deeper into the AC than in sample 298-1. Similar differences in collagen type II and aggrecan deposition were observed among AC samples classified as MG-2 (data not shown).

A broader range of IHC staining patterns was observed within MG-3 graded AC samples compared to either MG-1 or MG-2 samples. For example, samples 268-2 and 272-3 displayed significant differences in both the distribution pattern and extent of collagen type II and aggrecan staining despite comparable levels of disruption in the superficial layer (Fig. 4.3a,b) and similar losses in proteoglycan staining (Fig. 4.3c,d). Punctuate staining for collagen type II was observed in the superficial and transitional layers of 272-3 (Fig. 4.3f) whereas the corresponding region in samples 268-2 (Fig. 4.3e) was virtually devoid of staining. Sample 268-2 also exhibited a far more profound loss of aggrecan staining in the territorial regions of the transitional and deep layers (Fig. 4.3g) when compared to sample 272-3 (Fig 4.3h). Interestingly, these marked IHC differences in aggrecan deposition were observed in AC samples with histologically comparable levels of proteoglycan staining. MG-3 and MG-4 samples (data not shown) displayed a similar range of IHC differences for both collagen type II and aggrecan.

The most visually striking IHC differences were observed within MG-5 AC samples. For example, samples 272-1 and 225-1 displayed profoundly different staining patterns for both collagen type II and aggrecan. Collagen type II staining in sample 225-1 (Fig. 4.4f) was relatively homogeneous in the upper half of the AC section, while sample 272-1 exhibited a marked decrease in staining throughout the territorial region (Fig. 4.4e). In sample 272-1, aggrecan staining in

the interterritorial region was very intense from the superficial to the deep layer (Fig. 4.4g). Conversely, the superficial and transitional layers in sample 225-1 were nearly devoid of aggrecan staining (Fig. 4.4h). In addition to displaying the most visually striking IHC differences, MG-5 samples also displayed some of the most divergent staining of any grade examined, with 4 of 6 samples showing IHC staining patterns which were unique within the group.

4.4 Discussion

This was a descriptive study that summarized the IHC findings for 30 OA AC samples, spanning MG 1-5. IHC differences in collagen type II and aggrecan between AC samples of the same MG were examined. This study provided evidence that molecular differences exist within histological grades that could constitute novel subcategories within the established histological progression of OA.

Traditional histological scoring of AC failed to reveal differences in disease progression within histological grades that were detected at the IHC level. Even among AC samples representing the earliest stage of OA (MG-1), differences were observed in the extent of collagen type II and aggrecan staining. Because OA related changes begin at the articular surface and proceed deeper with disease progression (29), the greater loss of aggrecan from the transitional layer of sample 300-1 indicated that it represented a more advanced stage of OA than sample 298-1. The distinction between these MG-1 graded samples at the IHC level suggested that a more detailed description and categorization of early OA progression is possible and that molecular subcategories may exist within each of the histological grades. Furthermore, the IHC detection of such early molecular changes also supported and extended the findings of a recent study that showed molecular changes in OA occurred well before any signs of overt structural had damage emerged(30).

When MG-3 samples of AC were examined, significant differences in the depth and distribution of staining were observed for both collagen type II and aggrecan. Within these samples, changes in collagen type II distribution were observed that were consistent with the change in collagen type II metabolism reported elsewhere (10). While the progressive loss of collagen type II from the superficial and transitional layers is characteristic of the earliest stages of AC deterioration, MG-3 appeared to represent a turning point in the expression of collagen II. Interestingly, the appearance of collagen type II throughout the transitional layer was observed in some MG-3 samples, but not in others. For example, the punctuate, territorial collagen type II staining seen in the transitional layer in Fig. 4.3f, which appeared to mark a return of collagen type II to this region, was not observed in Fig. 4.3e. Judging from the absence of collagen type II in the superficial layer of sample 268-2, it represented an earlier catabolic phase in OA progression, while the punctate territorial staining seen in the superficial layer of 272-3 suggested that it may have recently entered an anabolic phase. This pronounced difference in the distribution of collagen staining suggested that IHC techniques can detect evidence of a change in metabolism within samples of the same histological grade. The IHC variability observed within MG-3 samples provided further evidence for the existence of subcategories within distinct histological grades.

In samples graded higher than MG-3 considerable IHC variability in collagen II and aggrecan deposition was observed between samples of the same histological grade. For example, MG-5 samples such as 272-1 and 225-1 displayed profound differences in the staining pattern and overall distribution of both of these extracellular matrix components. Given the divergent patterns of staining for collagen type II and aggrecan among MG-5 samples, it was difficult to conceive how these could have represented subsequent subcategories within a simple model of linear cartilage deterioration. Since these samples have been collected from a variety of different patients with varying degrees of joint involvement and because AC is known to undergo changes in metabolism with disease progression,

it was likely that the complex array of staining patterns observed in MG-5 AC arose as the result of these factors. In fact, this assumption was supported in part by the histological observations leading to the MG itself. For example, while both samples (272-1 and 225-1) obtained a score of MG-5, they arrived at this grade based on the additive scoring of such diverse histological characteristics as tissue structure, extent of safranin-O staining and cellularity. Sample 272-1 received component scores of: 2 for structure; 1 for safranin-O staining; 2 for cellularity, while sample 225-1 received scores of: 3 for structure; 2 for safranin-O staining; 0 for cellularity. Despite being the same MG, the differences in the individual component scores suggested that these samples had undergone significantly different cellular and proteolytic processes leading up to the point of surgery. Clearly all three of the characteristics, on which current histological scoring systems are based, are not associated with IHC staining to the same degree. As a result, the MG alone should not be relied upon to predict the depth or distribution of ECM components such collagen type II and aggrecan in more advanced OA samples.

Taken together these findings suggested that in addition to the more detailed description of OA progression afforded by IHC staining, staining for aggrecan may represent a sensitive indicator of very early disease progression whereas collagen type II staining may serve as an indicator of metabolic status of AC. Finally, we concluded that the use of the Mankin Scoring System can be misleading because AC samples of the same histologic grade can display significantly different molecular profiles and histological staining alone lacks the appropriate resolution to distinguish between them. The IHC disparities seen in MG-1 through MG-5 underscored the need for further molecular characterization in early AC degeneration. Additionally, because no IHC scoring system exists for AC, either a new scoring system would need to be developed or an IHC module would need to be incorporated into an existing scoring system to enhance the understanding of early AC degeneration. It is conceivable that once developed, an IHC scoring system for human AC could not only be readily adapted to animal

models of human OA but perhaps also modified to assess the quality of tissue-engineered AC.

Figures

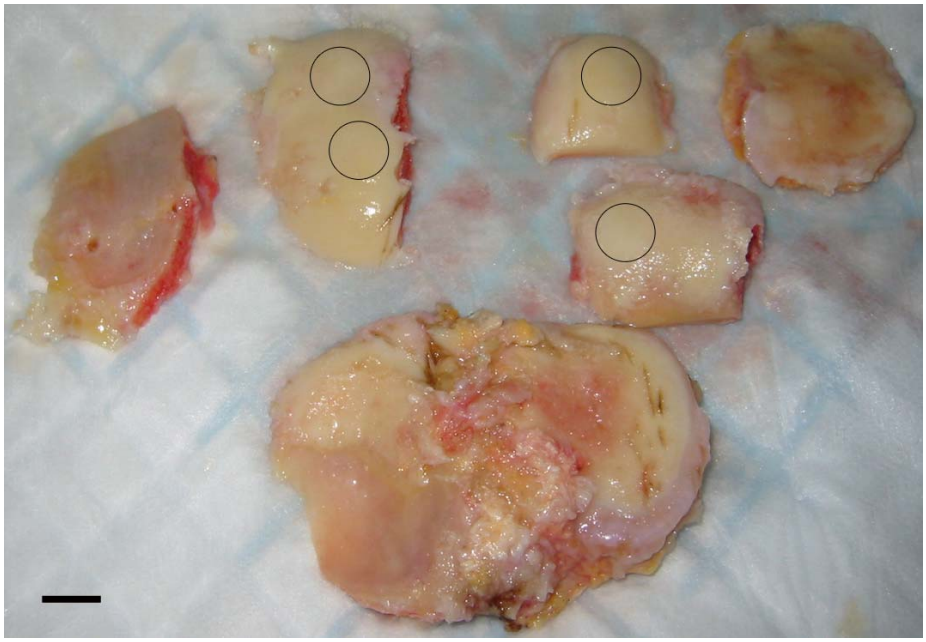

Figure 4.1. Image showing typical OA tissue obtained from patients undergoing total knee arthroplasty. Circles indicate the potential sites for collection of 1 cm diameter dowel in this specimen. Bar = 1 cm.

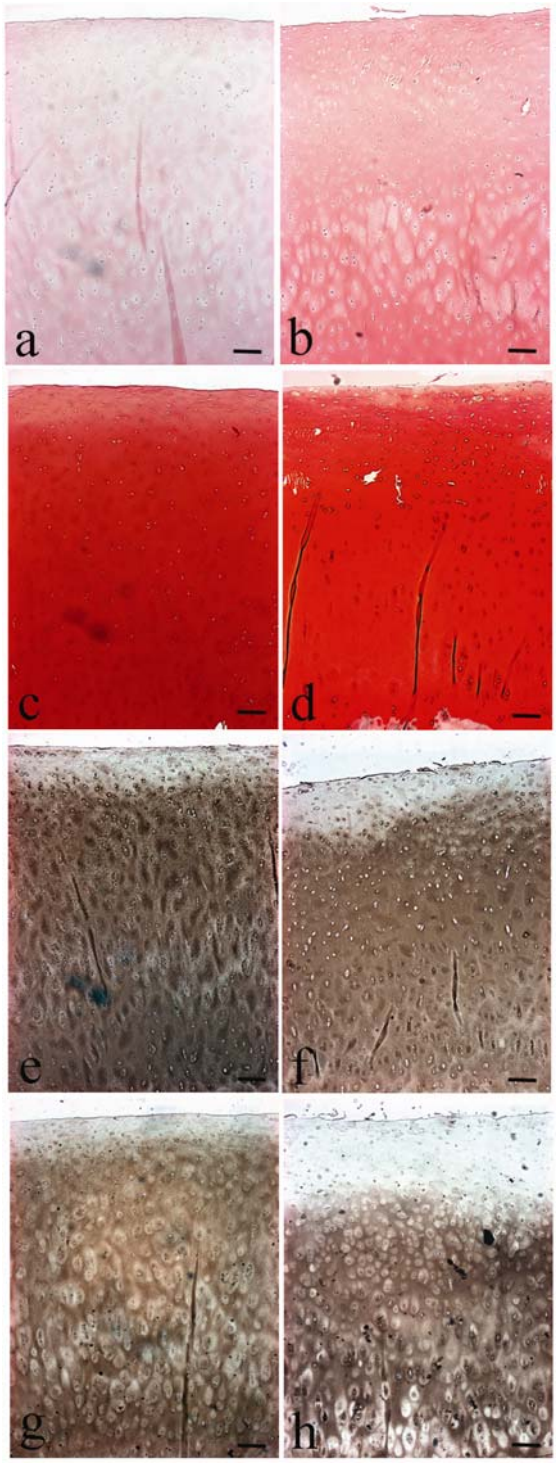

Figure 4.2. Micrograph of MG-1 graded OA sections of AC (298-1 and 300-1) showing histological staining for H&E (a,b) and safranin-O (c,d) as well as IHC staining for collagen type II (e,f) and aggrecan (g,h). Note the differences in the distribution of staining for collagen type II and aggrecan within each pair. Bar = 100 μm.

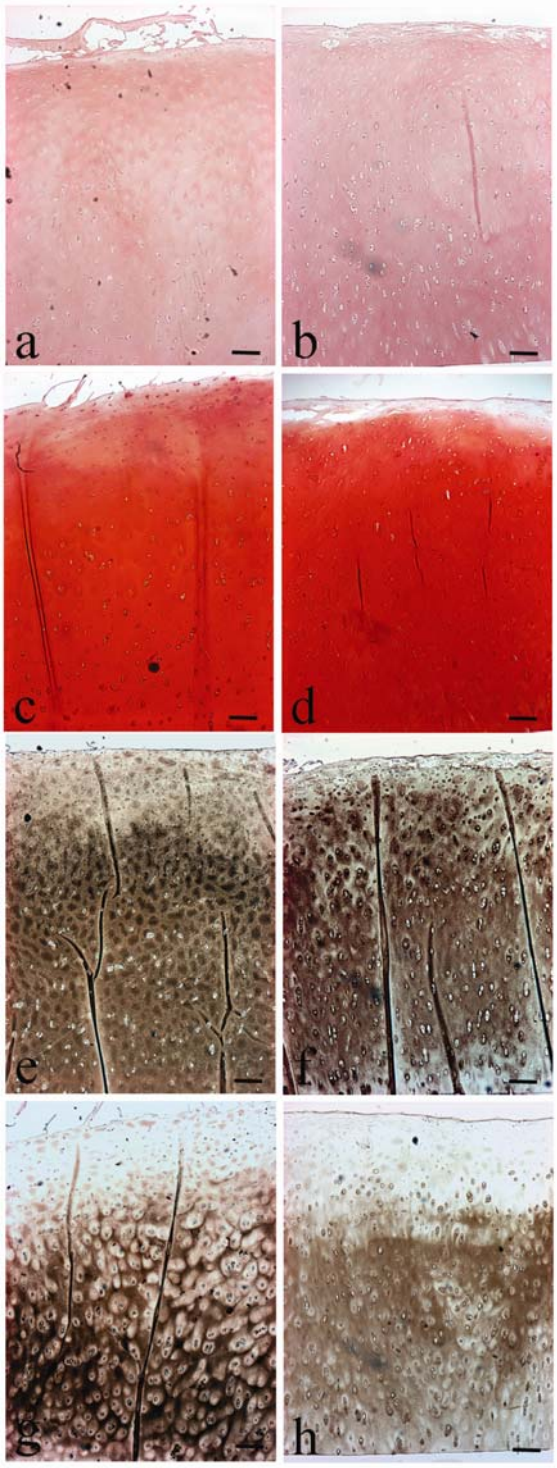

Figure 4.3. Micrograph of MG-3 graded OA sections of AC (268-2 and 272-3) showing histological staining for H&E (a,b) and safranin-O (c,d) as well as IHC staining for collagen type II (e,f) and aggrecan (g,h). Note the differences in the distribution of staining for collagen type II and aggrecan within each pair. Bar = 100 μm.

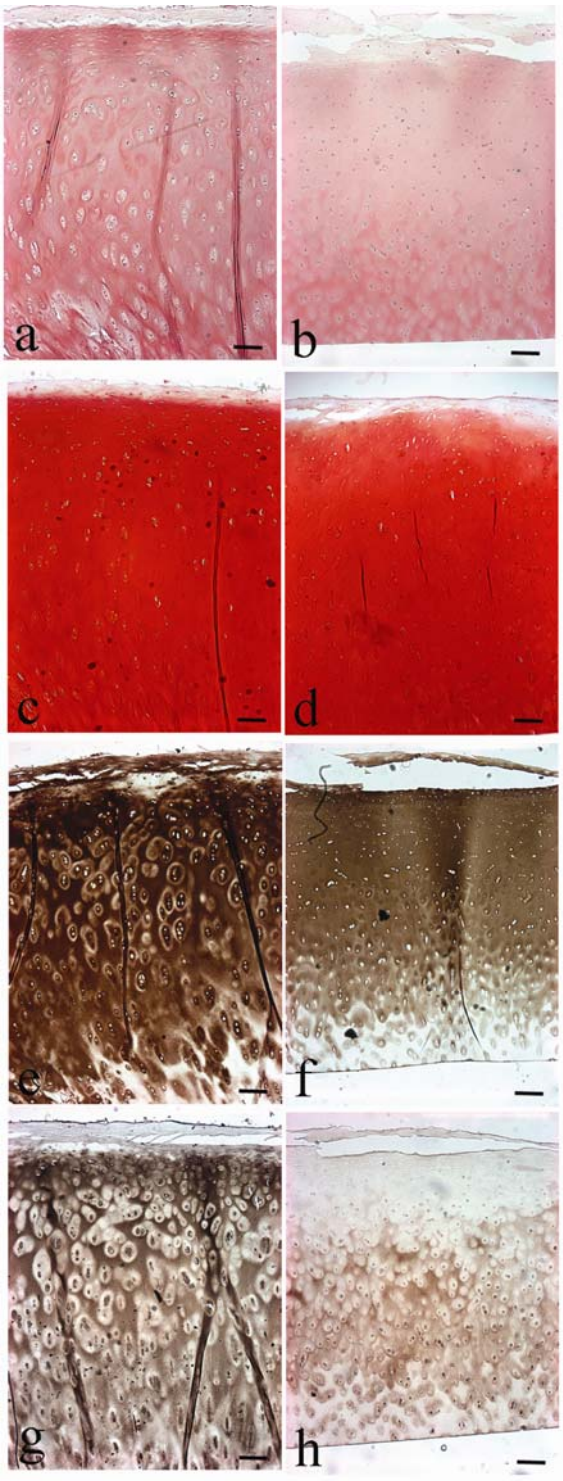

Figure 4.4. Micrograph of MG-5 graded OA sections of AC (272-1 and 225-1) showing histological staining for H&E (a,b) and safranin-O (c,d) as well as IHC staining for collagen type II (e,f) and aggrecan (g,h). Note the differences in the distribution of staining for collagen type II and aggrecan within each pair. Bar = 100 μm.

Table 4.1. Summary of the modified scoring criteria for the Mankin classification system.

I.	**Structure**		**II. Cells**	
	a. Normal	0	a. Normal	0
	b. Surface irregularities	1	b. Diffuse hypercellularity	1
	c. Pannus + irregularities	2	c. Cloning	2
	d. Clefts to transitional	3	d. Hypocellularity	3
	e. Clefts to radial	4		
	f. Clefts to calcified	5		
	g. Disorganization	6		
III.	**Safranin-O staining**			
	a. Normal	0		
	b. Slight reduction	1		
	c. Moderate reduction	2		
	d. Severe reduction	3		
	e. No dye noted	4		

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CHAPTER FIVE: Immunohistochemical Characterization of D*e Novo* Cartilage Present in Human Osteoarthritic Tissue. [@]

5.1 Introduction

Osteoarthritis (OA) is a common, degenerative joint disease characterized by pain, joint stiffness and dysfunction which represents a major cause of disability (1). Although traditionally viewed as a continuously progressive degenerative disease (2), evidence continues to emerge which details the non-linearity of OA progression. For example, 23% of OA patients report periods of symptomatic improvement (3) suggesting that interludes in disease progression may be a common occurrence. The complexity of OA progression appears to extend beyond mere interludes between periods of active cartilage erosion, because physical evidence indicates that 16% of patients with OA of the knee demonstrate radiographic increases in their joint space (4). While the precise type of tissue responsible for the above-mentioned increases remains unclear, a more recent study by Shibakawa *et al*. found that the *de novo* tissue within OA joints closely resembles fibrocartilage (5). When taken together, these reports suggest that interludes exist during OA progression in which the production of repair-like fibrocartilage may be a common feature. Despite the mounting evidence for the production of *de novo* tissue during OA progression, the origins of this tissue, the mechanisms underlying its development and the molecular composition remain unclear.

There are two general categories of *de novo* cartilage that have been described in association with advanced OA: fibrocartilage (6) and osteophytes (7). According to one study, fibrocartilage occurs in over 90% of total knee arthroplasty (TKA) specimens, thus indicating the ubiquitous intrinsic repair capacity of AC (8). This abundant repair tissue, which typically overlays damaged articular cartilage (AC) in OA joints, was termed pannus-like by Shibakawa *et al*. in order to distinguish it from true pannus tissue observed in rheumatoid arthritis (5). In contrast to the expression profile seen in AC, Shibakawa *et al*. reported that the pannus-like tissue was high in collagen type I and low in both aggrecan and collagen type II.

@ May be accepted by the journal Virchows Archiv pending revisions

Despite the prevalence of fibrocartilage in OA, very few studies have examined the structure and composition this *de novo* tissue in OA. In contrast to the pannus-like fibrocartilage, osteophytes are cartilage-covered nodules of bone that characteristically appear around the margins of osteoarthritic joints (reviewed in (9). Despite being a hallmark of OA, osteophytes also have not been widely studied at either the cellular or molecular levels. One study on osteophyte development conducted by Gelse *et al*. found that osteophytes begin as fibrous tissue and mature through a series of steps ultimately resulting in cartilage with a distinctly hyaline-like appearance (10). They discovered that juvenile osteophytes are high in collagen type I and low in aggrecan and collagen type II (ie. similar to fibrocartilage), whereas mature osteophytes were low in collagen type I and high in aggrecan and collagen type II (ie. similar to hyaline cartilage). Given the similarities between juvenile osteophytes and the pannus like tissue reported by Shibakawa *et al*. a direct immunohistological comparison is warranted to determine the extent of the similarities and whether there is also evidence for maturation among samples of pannus-like tissue.

Given that *de novo* tissues are a common, yet frequently overlooked feature of OA, this study was designed to ascertain 1) whether *de novo* fibrocartilagenous tissue was present in minimally damaged AC, 2) the extent to which *de novo* fibrocartilagenous tissues share IHC features in common with osteophytes, and 3) whether *de novo* tissues associated with early AC deterioration displayed the typical pattern of ECM expression for fibrocartilage. This study provides a descriptive histological and immunohistochemical analysis and comparison of the *de novo* tissue associated with OA and explores the role of intrinsic repair in OA progression.

5.2 Materials and Methods

5.2.1 Tissue Collection and Processing

OA tissue from femoral condyles was obtained during surgery for TKA. The tissue was immediately placed in saline solution and stored at 4°C for up to 24

hrs. One centimeter diameter cylindrical cores of AC and underlying bone (dowels) were obtained from load-bearing regions using a stainless steel coring tool. Cartilage was removed from the underlying bone at the cartilage-bone junction and the AC was fixed in 4% formalin. Dowels representing only Outerbridge (OB) grades 0, 1 and 2 (11) were collected for analysis. A total of ten dowels were collected for each of the three OB grades for a total of 30 dowels. The 30 dowels were collected from 17 OA patients (7 males and 10 females) that ranged in age from 58 to 84 years with a mean age of 67.4 +/- 7.4 years. The second experimental group consisted of three fully-developed, mature looking osteophytes that were harvested from within a single joint. The osteophyte cartilage was removed from the underlying bone and this tissue was treated in parallel with the cartilage from the dowels.

5.2.2 Histology

Formalin-fixed AC was embedded in paraffin, sectioned at 5 μm intervals and mounted on Superfrost Plus glass slides. Immediately prior to staining, sections were deparaffinized using ProPar (Anatech Ltd) and rehydrated in a graded series of ethanol washes. Deparaffinized sections were subjected to routine hematoxylin and eosin (H&E) staining to show overall tissue morphology. All sections were dehydrated through a series of graded ethanol washes for 20 seconds each, mounted and visualized at 50x magnification on a Leica DM/RE microscope. Photomicrographs of representative regions within the sections were taken using a CoolSnap CCD camera (Fig. 5.1). All sections were visually examined and the presence of any fibrous tissue or fibrocartilage was noted.

5.2.3 Immunohistochemistry

AC sections were deparaffinized and subjected to an optimized antigen retrieval step consisting of a 60 min digestion in 5 mg/ml pepsin, pH 2.0 (Sigma Cat # P-7000). The pepsin digest was optimized to avoid saturation staining and thus yield the greatest amount of information regarding relative staining levels within a section. Following digestion, sections were rinsed thoroughly in water to remove

the pepsin and endogenous peroxidase activity was quenched using a 30 min incubation in 3% H_2O_2 in methanol. All immunohistochemistry was performed using Vectastain Elite ABC kits (Vector Laboratories, Cat. # 6102 and 6105) according to the manufacturer's directions. Primary antibodies [mouse anti-collagen type I (MAB 3391, Chemicon) or mouse anti-collagen type II (MAB 8887, Chemicon) or rabbit anti-aggrecan (AF 1220, R&D Systems] were applied to separate AC sections from each sample at a 1:100 dilution in phosphate-buffered saline (PBS), then incubated for 30 min in a humidified chamber. Sections were washed in PBS and biotinylated secondary antibodies were applied for 30 min. Following the secondary antibody incubation, AC sections were washed in PBS and incubated for 30 min in VectaStain ABC reagent. After a final rinse in PBS, color development was achieved by incubating the sections for 5 min in metal-enhanced DAB (Pierce Cat # 1856090). Stained tissue sections were dehydrated using a series of graded ethanol washes, mounted and visualized at 50x magnification on a Leica DM/RE microscope. Photomicrographs of representative regions within the sections were taken using a CoolSnap CCD camera (Fig. 5.2 & 5.3). Confirmation of antibody specificity to collagen type I and collagen type II was performed using 5 μm thick sections of human anterior cruciate ligament (ACL). No cross-reactivity was observed with either MAB 3391 or MAB 8887. Sections in which primary antibodies were omitted showed no non-specific staining.

5.3 Results

5.3.1 Histology

Three of the thirty samples displayed morphology atypical for either normal AC or degraded OA cartilage. This atypical tissue ranged in appearance from fibrous tissue (a high density of elongated fibroblast-like cells in a matrix displaying the typical wavy pattern of collagen fibril staining) to fibrocartilage (round, sparsely distributed chondrocyte-like cells in a more homogeneously staining matrix). In all three cases, the *de novo* tissue was associated with AC samples showing some of the most advanced signs of deterioration in the study (i.e. Outerbridge 2). Fig.

5.1a shows an example of characteristic fibrous tissue in-filling a large, deep fissure in the native AC. Integration of the fibrous tissue appeared to be very good; however, the defect was not completely filled and an irregular surface remained. Fig. 5.1b shows an example of tissue more closely resembling fibrocartilage which had resurfaced an area larger than the dowel itself. The incompletely integrated pannus-like tissue loosely overlaid the previously eroded native AC. In contrast to the irregular surface associated with the fibrous tissue, the sample in Fig. 5.1b yielded a relatively smooth surface. Finally, Fig. 5.1c provided an example of fibrocartilage greater than 0.5 mm thick that was observed covering a region larger than 1 cm in diameter. This *de novo* tissue was completely integrated with the underlying native AC below as evidenced by the sharply delineated, yet congruent boundary between the upper fibrocartilage and lower AC. Unlike the pannus often associated with rheumatoid arthritis tissue, the pannus-like tissue in this study appeared to be avascular.

Similar to the pannus-like tissue, the histology of osteophyte cartilage also displayed a broad range of tissue types. Osteophyte cartilage ranged from a heterogeneous mixture of fibrous tissue (Fig. 5.1d) to tissue closely resembling hyaline cartilage (Fig. 5.1f). The overall level of tissue organization differed considerably both within and between samples. Histologically, the surface of the osteophytes varied from relatively smooth (Fig. 5.1e) to moderately fibrillated (Fig. 5.1d). The average depth of osteophyte cartilage (1.84 ± 0.10 mm) was consistent with the depth of observed in normal human knee AC (12).

5.3.2 Immunohistochemistry

Abundant collagen type II staining was detected in fibrocartilagenous tissue regardless of the degree of tissue integration. When compared to the underlying native AC, intermediate to high levels of collagen type II staining were observed throughout the fibrocartilagenous tissue (Fig. 5.2b,c). However, differences were observed in the pattern of collagen type II staining, between incompletely integrated and well-integrated samples, despite the similarity in their overall

levels of collagen type II deposition. Unlike the incompletely integrated pannus-like tissue, the well-integrated tissue displayed the type of territorial collagen type II deposition that is characteristic of AC in the early stages of OA. In contrast to the fibrocartilagenous tissue, the fibrous tissue was nearly devoid of collagen type II staining (Fig. 5.2a). Aggrecan was abundant in all three pannus-like samples and the level of staining was comparable to the levels seen in the underlying native AC (Fig. 5.2d-f).

The pattern of aggrecan distribution in the well-integrated pannus-like tissue (Fig. 5.2f) more closely resembled the homogeneous staining typically seen in AC, whereas the staining in the incompletely integrated pannus-like tissue possessed a granular appearance (Fig. 5.2e). Like the aggrecan staining, collagen type I staining was observed in all three pannus-like tissue samples (Fig. 5.2g-i). The pannus-like tissue displayed levels of collagen type I staining comparable to the levels seen in the immediately adjacent underlying native AC; with minimal collage type I noted in the deeper layers.

The osteophytes exhibited an ECM expression profile that was similar to the profile observed in the pannus-like tissue. All three samples of osteophyte cartilage displayed pronounced collagen type II staining (Fig. 5.3a-c). However, there was considerable variability in this staining both within and between the samples. The samples displayed evidence of the territorial localization of collagen type II typically seen in AC during the early to intermediate stages of OA (13). A broad range of staining for aggrecan was also observed between osteophyte cartilage samples (Fig. 5.3d-f). Although aggrecan was present in all osteophyte cartilage samples, one sample (Fig. 5.3d) displayed a highly irregular aggrecan distribution. This sample contained large regions that were nearly devoid of aggrecan staining into which sharply-defined, intensely-staining, aggrecan projections were interspersed. This was in contrast to the other two samples (Fig. 5.3e, f) which stained relatively evenly from the superficial to deep layers. The intensely staining osteophyte samples (Fig 5.3e, f) showed a reduction in

territorial staining for aggrecan that is typically associated with AC in the early stages of OA (14). Finally, osteophyte cartilage also displayed a broad range of collagen type I deposition (Fig. 5.3g-i). Although some collagen type I deposition was found throughout the section displaying deep fissuring (Fig. 5.3g) it was predominantly associated with the superficial-most layer of osteophyte cartilage. Interestingly, the depth of collagen type I staining in the sections appeared to increase as a function of structural damage.

5.4 Discussion

The quality of some of the *de novo* cartilage observed in this study demonstrated the considerable potential of intrinsic repair that exists in OA tissue. The pannus-like tissue varied from fibrous tissue which occupied the spaces between deep fissures in the eroded AC, to a thick layer of fibrocartilage which restored a relatively smooth surface to the previously eroded area. The extent of pannus-like tissue integration also varied considerably but good integration was observed in cases of both fibrous tissue and fibrocartilage.

5.4.1 Pannus-Like Tissue

Histologically the pannus-like repair tissue did not possess the structural organization characteristics of normal or osteoarthritic AC, but it did share some of the immunohistochemical characteristics. For example, staining for collagen type II and aggrecan were surprisingly abundant, which is in contrast to the very low levels expected in fibrocartilage. These observations suggest that while repair tissue may lack the structural organization of AC, it can display an abundance of the two hallmark extracellular matrix (ECM) components that define AC. This finding is in contrast to the findings by Shibakawa *et al*. (5) who reported that pannus-like tissue showed elevated staining for collagen type I and was devoid of staining for both proteoglycans and collagen type II. Although such divergent findings are difficult to explain, Shibakawa *et al*. included vascular pannus-like tissue in their study, which likely has different origins and hence gene expression patterns form the avascular pannus-like tissue examined here.

5.4.2 Structural AC Damage and Repair

Based on the distribution of *de novo* tissue within this study there appeared to be a relationship between structural damage and the appearance of pannus-like tissue. In the 30 samples of OA tissue that ranged from OB-0 to OB-2, evidence of repair tissue was present in only three of them. Although one of the three samples was initially scored as OB-1, upon histological inspection it became apparent that the tissue actually displayed evidence of damage extending into the transitional layer that was consistent with a score of OB-2. Interestingly, this damage was effectively masked by a layer of fibrocartilage that yielded a remarkably smooth surface and resulted in the underestimation of the actual AC deterioration. Even though all samples were obtained from patients undergoing TKA for advanced OA, *de novo* tissue was not found in AC with levels of tissue deterioration consistent with either OB-0 or OB-1. The association between structural damage and *de novo* tissue synthesis suggested that tissue repair may be a localized cellular response only initiated by conditions such as structural damage or apoptosis that are common in the intermediate to late stages of OA(15). Interestingly, the elevated anabolism that coincides with the emergence of structural damage (16) may be directly responsible for the production of the *de novo* tissue seen in intermediate stages of OA. This surge in anabolism may temporarily permit the AC to overcome the net loss of ECM components and the subsequent accumulation of ECM in the superficial zone could explain the observed appearance of repair tissue in structurally damaged AC. Furthermore the accumulation of this repair tissue, could account for both the improvement in patient symptoms reviewed in Buckwalter *et al*. (2) and the reported radiographic increases in joint space and thus may represent part of the normal course of disease progression in OA.

5.4.3 Development of Pannus-Like Tissue

The origin of this pannus-like tissue in OA could arise through one of two distinct mechanisms. While it is possible that existing native AC may have been remodeled into fibrocartilage as Shibakawa *et al*. (5) have speculated, it is more

likely that the fibrocartilage in Fig. 5.1c represents a *de novo* cartilage synthesis event. Simple remodeling of the tissue would not account for the sudden contrast in cellular distribution or the sharply delineated boundary that exist between upper fibrocartilage and the underlying native AC. Furthermore, the fact that both collagen type I and II have recently been shown to accumulate in very high levels in moderately fibrillated tissue (16) indicates that the step preceding the emergence of fibrocartilage displays the appropriate precursory levels of anabolism for the generation of *de novo* tissue. Therefore, it is likely that the observed *de novo* synthesis is merely an extension of the natural, albeit short lasting, anabolic interlude that has been described in early to intermediate OA (17-21).

5.4.4 Osteophytes and De Novo Cartilage

Osteophytes were examined alongside pannus-like tissue to explore histological and immunohistochemical parallels between these *de novo* tissues. Osteophyte cartilage appeared to have a surprising amount in common with the pannus-like fibrocartilage at the histological level. These commonalities also extended to the IHC level, because the two types of tissues shared a similar expression profile for collagen type I, collagen type II and aggrecan. Given the organizational, histological and IHC similarities between the cartilage associated with osteophytes and the pannus-like tissue, their development likely relies on many of the same genetic and biochemical signaling pathways. If this is true, then perhaps the pannus-like cartilage that develops in OA joints could share the same pattern of maturation as osteophyites (if provided the appropriate environment) as described by Gelse *et al*. (10). In this model, pannus-like tissue would begin as fibrous tissue devoid of collagen type II and aggrecan and high in collagen type I (much like what was described by Shibakawa) and progress through several stages until the tissue resembled well integrated fibrocartilage high in collagen type II and aggrecan (as described here). In fact, Koshino *et al.* (22) indirectly provided evidence for a range of maturity levels in their study on the regeneration of AC following high tibial osteotomy. They described a series of 47 eburnated

OA joints that were intrinsically resurfaced yielding a range of tissue types all the way up to hyaline-like cartilage. The Koshino *et al.* study supports the concept that *pannus-like* repair tissue matures through a series of stages much like osteophytes and is further evidence of the commonly overlooked yet significant repair potential that exists in OA cartilage.

Finally, given that Gelse *et al.* (10) found that the cartilage of fully mature osteophytes very closely resembles hyaline cartilage, it is possible that the pannus-like tissue also has this potential. For this reason, the knowledge gained from a closer examination of the intrinsic repair process and the factors leading to its ultimate failure would be invaluable towards our understanding of the natural barriers that are currently impeding extrinsic repair techniques.

In this study, pannus-like tissue ranging from fibrous tissue to fibrocartilage was associated with structurally damaged OA cartilage. The *pannus-like* tissue was not observed in samples exhibiting the lower levels of deterioration seen in OB grades 0 or 1 suggesting that the intrinsic repair process may be highly localized (i.e. OB 0 and 1 samples did not have evidence of pannus-like tissue when OB 2 samples from the *same* joint did). Given that OB-2 tissue typically displays elevated levels of anabolism and that *de novo* tissue was not observed in the predominantly catabolic OB-1 samples, it is likely that *de novo* synthesis is a direct extension of the anabolic interlude seen in later stages of OA. Finally, the osteophyte associated cartilage and pannus-like fibrocartilage showed numerous similarities at the histological and IHC levels suggesting they may share a common evolution.

Figures

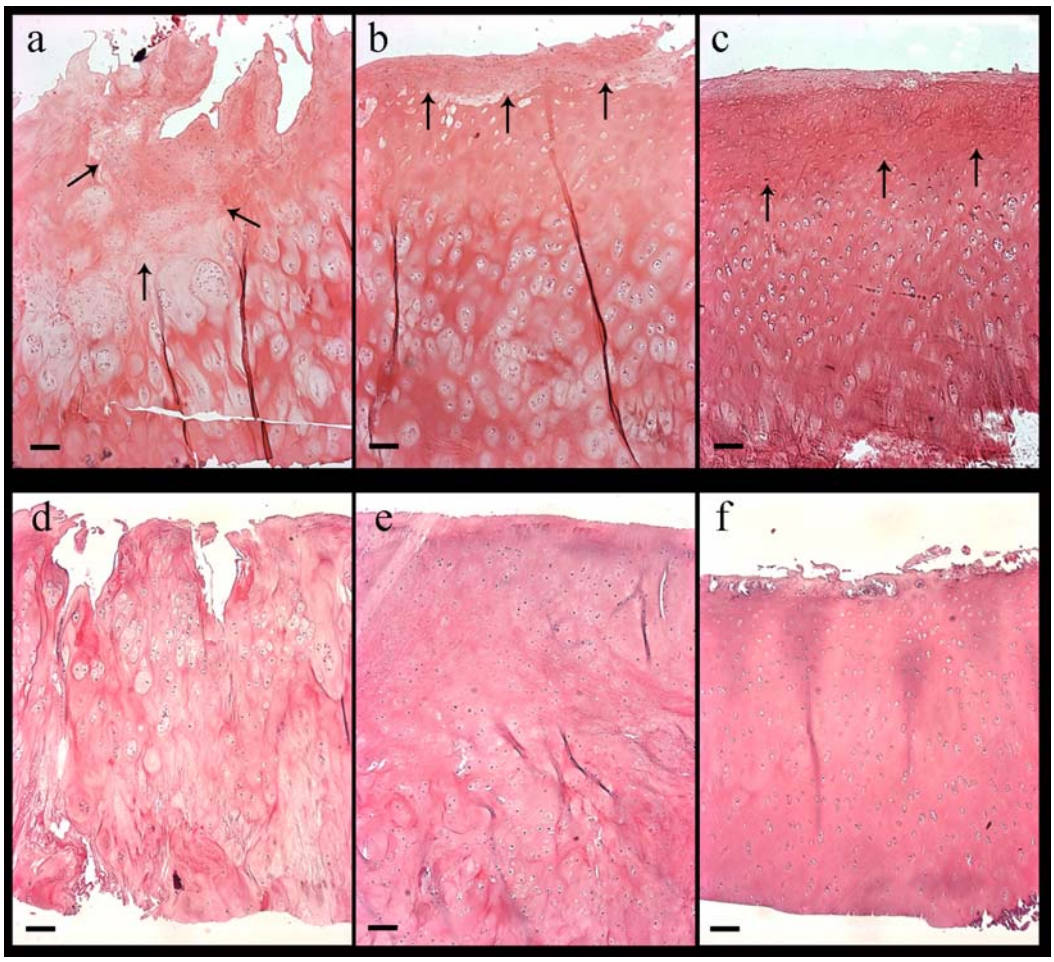

Figure 5.1. Micrographs showing the range of pannus-like tissue (a-c) and osteophyte cartilage (d-f) found in osteoarthritis specimens. Hematoxylin and eosin stained sections showing; (a) a well integrated fibrous repair tissue, (b) a poorly integrated fibrocartilagenous lamination, and (c) a fibrocartilagenous restoration of an articulating surface, (d) a mixture of fibrous tissue and fibrocartilage, (e) predominantly fibrocartilage, and (f) predominantly hyaline-like tissue. Tissue below the arrows represents native articular cartilage while tissue above the arrows represents *de novo* tissue synthesis. Notice the morphological variation within both the pannus-like tissue and osteophyte cartilage. Bar = 100 μm.

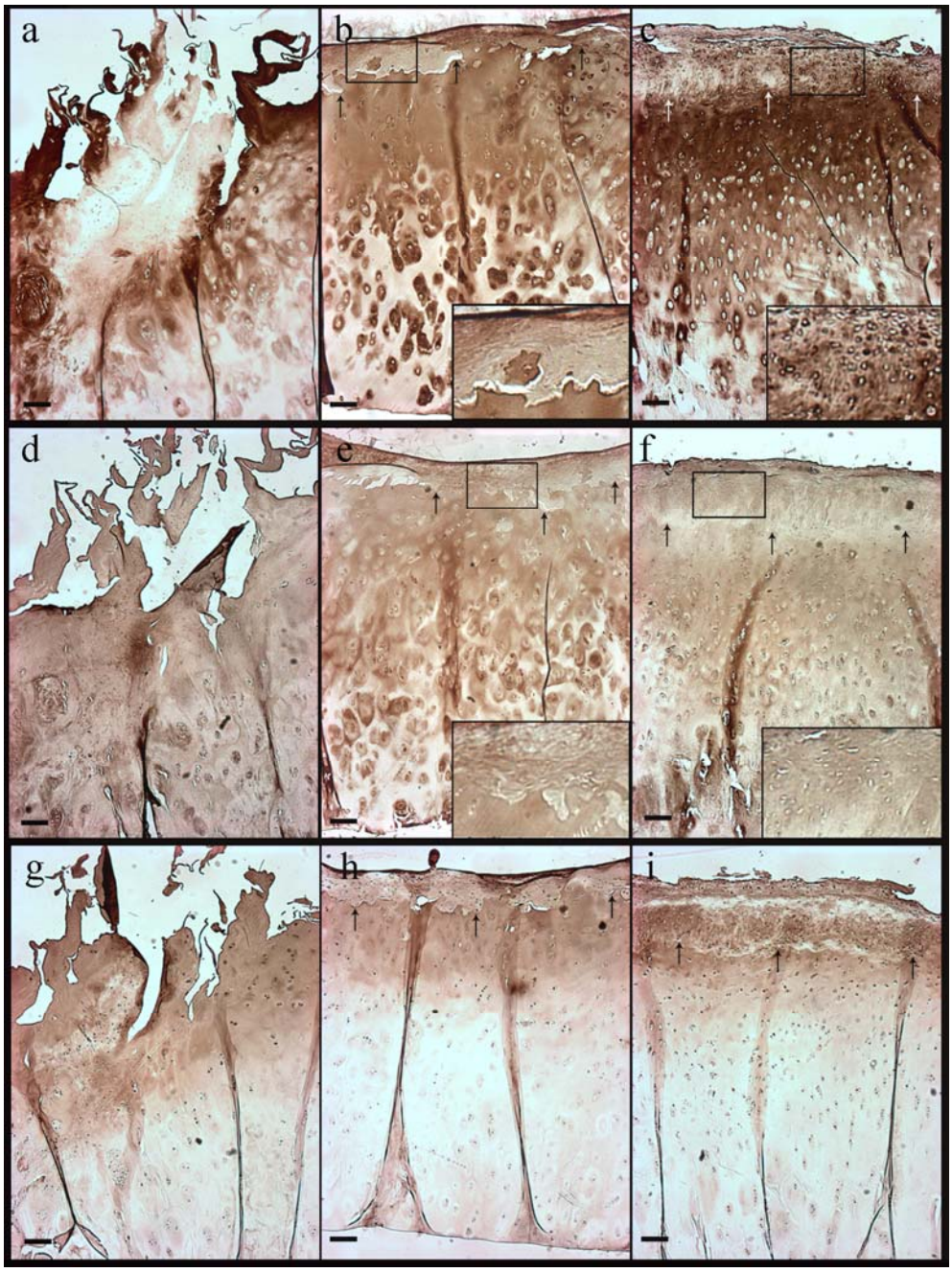

Figure 5.2. Micrographs showing pannus-like tissue sections stained for; collagen type II (a-c), aggrecan, (d-f), and collagen type I (g-i). Notice the abundance of collagen type II and aggrecan in the pannus-like tissue. Also note the strong similarities in the intensity of collagen type II and aggrecan staining between the pannus-like tissue and the native articular cartilage that underlies it. Arrows indicate *de novo* tissue boundary. Box indicates the location of the inset. Bar = 100 μm.

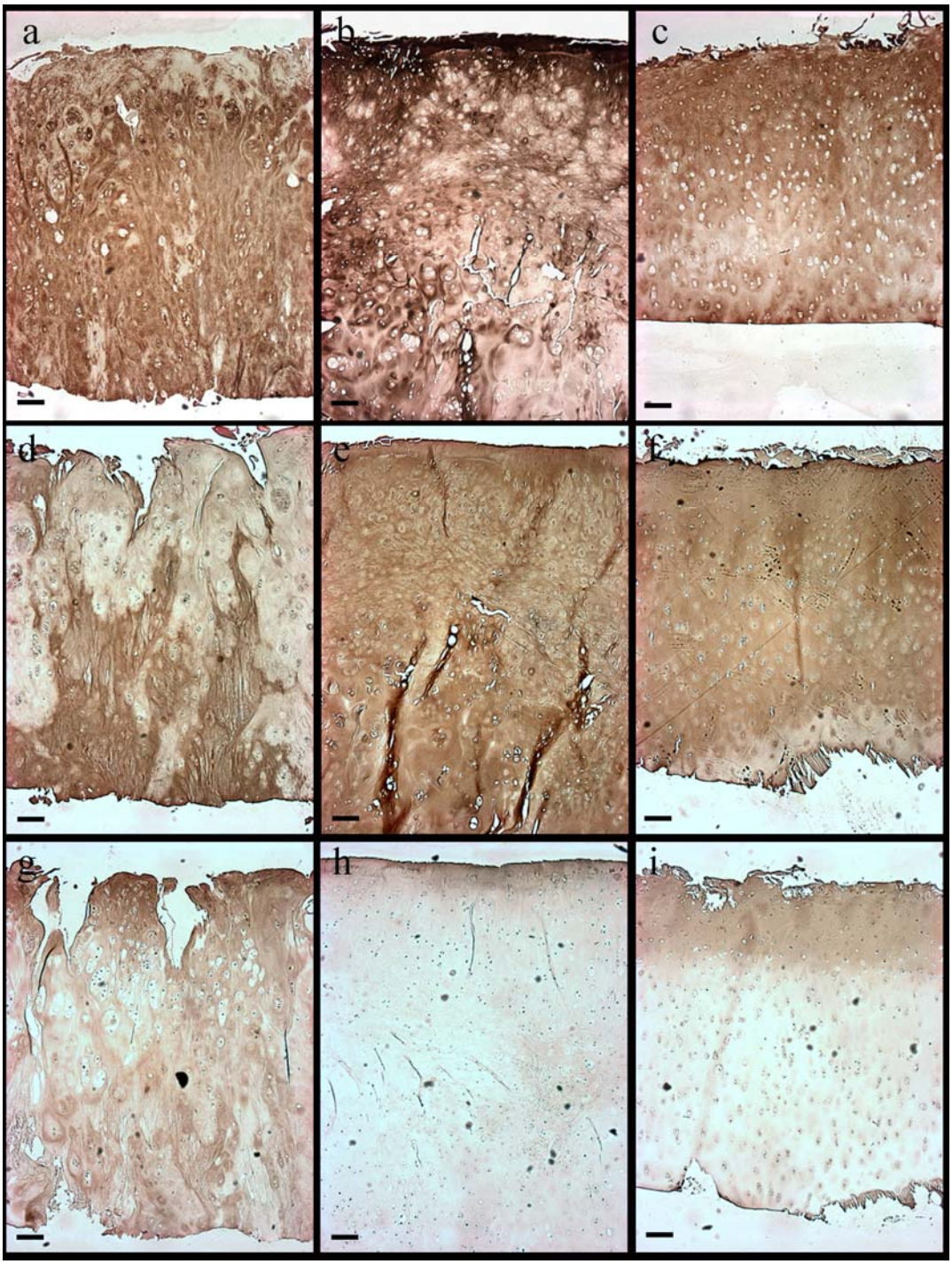

Figure 5.3. Micrographs showing the range of immunohistochemical staining for; collagen type II (a-c), aggrecan (d-f) and collagen type I (g-i) in serial sections taken from three human osteophytes. Note the abundant expression of collagen type II and aggrecan staining. Bar = 100 μm.

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CHAPTER SIX: Characterization of Novel Culture Conditions Which Prevent Articular Chondrocyte Dedifferentiation.

6.1 Introduction

6.1.1 Articular Cartilage and Hypoxia

Articular cartilage (AC) covers the ends of the long bones where it provides cushioning and reduces friction within a synovial joint. AC is an avascular tissue that receives all of its oxygen and nutrients via diffusion from the synovial fluid. As diffusion occurs from the articular surface through the AC, an oxygen concentration gradient is established within the tissue. Reports indicate that oxygen tension ranges from approximately 10% at the articular surface to 1% in the deepest layers of AC(1). While most cell types in the body are incapable of withstanding such limited O_2 availability, mounting evidence suggests that chondrocytes possess numerous subcellular adaptations to cope with their hypoxic environment. Until recently, most chondrocyte culture methods had ignored this important aspect.

6.1.2 Hypoxic Gene Regulation

The hypoxia inducible factor-1 (HIF-1) which has been extensively characterized in other systems has recently been implicated as a central transcription factor in the process of chondrogenesis(2). The importance of HIF-1 in chondrocytes has been further emphasized by additional reports which have implicated HIF-1 in a diverse array of essential chondrocyte processes including extracellular matrix (ECM) synthesis(3), cell proliferation(4), apoptosis(5) and glycolysis(6). HIF-1 is a heterodimeric complex consisting of a constitutively expressed oxygen sensitive subunit known as HIF-1$_\alpha$ and a nuclear translocation factor known as HIF-1$_\beta$(7). Under low oxygen tension, HIF-1$_\alpha$ translocates into the nucleus, associates with HIF-1$_\beta$ and binds to short DNA sequences known as hypoxia responsive elements (HRE)(8). Although HREs have been identified in a variety of genes including erythropoietin(9), transferrin(10), vascular endothelial growth factor(11), and glucose transporter-3(12), it remains unclear whether ECM genes such as collagen II and aggrecan are directly transcriptionally activated by HIF-1.

Another transcription factor known as SOX-9 has also been found to play a pivotal role in chondrogenesis(13). As such, SOX-9 may represent a molecular signaling intermediate between the oxygen sensing ability of HIF-1 and the transcriptional activation of ECM genes in chondrocytes. The *SOX-9* gene, which is transcriptionally activated by HIF-1(2), has itself been shown to be involved in the transcriptional activation of genes such as collagens II, IX and XI as well as aggrecan(13) and hence represents an excellent potential downstream modulator in HIF-1 induced maintenance of the chondrocyte phenotype. Appropriate activation of these hypoxia-inducible transcription factors should therefore be of paramount importance to *in-vitro*-based chondrocyte studies.

6.1.3 Dedifferentiation and Chondrocyte Culture

It is well established that chondrocytes in monolayer culture lose their phenotype(14). Studies have shown that there is a pronounced change in gene expression associated with chondrocyte dedifferentiation including a reduction in the expression of aggrecan(15) and collagen II(16) and an increase in expression of collagen I(16). Over the past three decades, many different culture conditions have been examined in an attempt to prevent the *in vitro* dedifferentiation of chondrocytes. For example, studies have examined phenotypic stability of chondrocytes using high density monolayer culture(17), pellet culture(18), alginate bead encapsulation(19), agarose gels(20), bioreactors(21), cytokines(22-24) and, most recently, hypoxia(25). However, while it is generally accepted that some elements of the chondrocyte phenotype can either be preserved or promoted through the use of these individual techniques, no single technique seems able to fully preserve all elements(26,27).

6.1.4 Tissue Engineering & Dedifferentiation

Recently, innovative cell-based, extrinsic repair techniques such as autologous chondrocyte implantation (ACI)(28) and matrix-induced chondrocyte implantation (MACI)(29) have been developed to repair acute chondral defects. Unfortunately, a major problem with these approaches is that they predominantly

yield fibrocartilage rather than the hyaline cartilage that is characteristic of AC(30). Given that these techniques rely on the *ex vivo* propagation of chondrocytes, under non-physiologic conditions which have been shown to cause dedifferentiation, it is perhaps not surprising that the tissue produced by these fibroblast-like cells more closely resembles fibrocartilage than hyaline cartilage *in vitro*. One way to improve the quality of extrinsic repair tissue would be to optimize *ex vivo* culture conditions to favor the retention of the chondrocytic phenotype but, to date, no study has identified such conditions that can successfully prevent the loss of appropriate ECM gene expression and sufficiently preserve phenotypic stability.

The objective of this study was to develop chondrocyte culture conditions that would permit the retention of the ECM gene expression patterns seen in freshly isolated human articular chondrocytes. To accomplish this, we compared gene expression levels obtained from high density monolayer chondrocyte (HDMC) cultures maintained under both normoxic (21% O_2) and hypoxic (5% O_2) conditions with values from similarly treated chondrocytes maintained under conventional 2D and 3D growth conditions. We postulated that sustained hypoxia in conjunction with an extended HDMC culture environment and appropriate cytokine supplementation would be sufficient to promote the retention of the chondrocytic phenotype in primary human articular chondrocytes.

6.2 Materials and Methods

6.2.1 Tissue Collection and Processing

Disease-free femoral condyles from a 19 year old female cadaveric organ donor were obtained through the Comprehensive Tissue Center and the Human Organ Procurement and Exchange (HOPE) program at the University of Alberta Hospital. The tissue was immediately rinsed in saline solution and full-depth AC was removed from the subchondral bone using a scalpel. AC fragments were digested for 6 hrs at 37°C using 1 mg/ml collagenase solution (Sigma-Aldrich, Oakville, ON) in DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 1x

Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA). Chondrocytes were recovered by centrifugation at 500g for 10 min and the cells were rinsed twice in phosphate-buffered saline (PBS) to remove residual collagenase. A small aliquot of freshly isolated chondrocytes (FIC) (~3 x 10^6) was immediately frozen at -80° C to be used as a positive gene expression control. The remaining chondrocytes were either plated at a density of 1 x 10^5 cells/cm^2 in Chondrocyte Growth Medium (CGM - CC-3216, Lonza, Basel, Switzerland) or encapsulated in alginate at a concentration of 2.5 x 10^6 cells/ml according to the procedure by Hauselmann *et al.*(31) and immersed in 3 volumes of Chondrocyte Differentiation Medium (CDM - CC-3225, Lonza, Basel, Switzerland). See Table 6.1 for details regarding media formulations.

6.2.2 Experimental Design

The gene expression patterns of three experimental groups were compared at two different oxygen tensions (5% and 20%) (Fig. 6.1). Primary chondrocyte cultures grown in CGM under normoxic conditions were either harvested upon reaching confluence (Group 1 - passage zero culture or P0) or were maintained in high density monolayer chondrocyte (Group 2 - HDMC) culture for 8 weeks, while the alginate-embedded chondrocyte (Group 3 - AEC) cultures were maintained for the same duration in CDM. To accommodate the elevated cell numbers in HDMC cultures, chondrocytes were maintained in 15 ml of medium per 100 mm culture dish. A parallel experiment was conducted under hypoxic conditions (5% O_2) using a ProOx meter (model 110) and associated C-chamber (C-374) (BioSpherix, Lacona, NY). Cultures were maintained in their respective humidified incubators at 37° C and media changes were performed biweekly for eight weeks. On the eighth week, cells were released from the alginate beads, washed in ice-cold PBS, centrifuged at 400 g for 5 min and frozen at -80°C. Prior to harvesting, P0 and HDMC cultures were washed twice with ice-cold PBS. Culture dishes were scraped with a rubber policeman to detach the cells and then cells were collected in a 15 ml conical tube and centrifuged at 400g for 5 min. The excess PBS was thoroughly aspirated from the pellets using a pipette whereby the wet weights

were measured and recorded prior to storage at -80°C. Three HDMC cultures were used to establish mean *de novo* tissue mass for hypoxic and normoxic treatments.

FICs were used as control cells, because all cell culture techniques, whether for clinical or basic science applications, depend on an initial step involving the isolation chondrocytes from the ECM that encases them. FICs represented positive control cells and GM38 fibroblasts grown under normoxic conditions served as negative control cells.

6.2.3 RNA Isolation, cDNA Synthesis and qPCR

RNA was purified from chondrocytes using the Biorad Aurum Total RNA fibrous tissue kit (732-6830, Biorad, Mississauga, ON) and quantified at 260 nm using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE.). Synthesis of cDNA was achieved using the iScript cDNA synthesis kit (170-8891, Biorad, Mississauga, ON). qPCR was performed on a MyiQ single-color real-time PCR machine (170-9770, Bio-Rad Laboratories, Mississagua, ON) using the primers listed in Table 6.2 in conjunction with the iQ SYBR Green Supermix (170-8882, Biorad, Mississauga, ON). The qPCR reaction was 45 cycles in length consisting of a 15 second denaturation step at 94°C; 20 second annealing step at the temp indicated in Table 6.2 and 10 second elongation step at 72°C. MyiQ optical software version 1.0 was used for the analysis of the reactions. All protocols were performed according to the manufacturer's recommendations. Comparisons between relative expression levels were conducted according to the $2^{-\Delta\Delta Ct}$ method(32) with GAPDH used the internal control gene.

6.2.4 Collagen and GAG Biosynthesis

For biochemical analyses, approximately 200 mg of each HDMC pellet was removed and placed in a microfuge tube containing three volumes of CyQuant (C7026, Invitrogen) cell lysis buffer. The samples were thawed on ice and thoroughly homogenized using 4 x 15 second pulses with a sonicator, then

RNAse treated for 1 hr at room temperature. DNA concentration in each of the samples was quantified according to the manufacture's recommendations using a PerkinElmer Victor *X3* series Multilabel Plate Reader. The amounts of collagen and sulfated GAGs in each sample were then determined using the Sircol (BioColor Ltd., Ireland) and Blyscan (Biocolor Ltd, Ireland.) assays respectively. Both assays were performed according to the manufacturer's recommendations and the resulting color development was measured using a Beckman DU640 spectrophotometer. The amounts of collagen and GAGs were normalized to the amount of DNA in each sample.

6.2.5 Statistical Analysis

A one-way analysis of variance (ANOVA) employing the Bonferroni multiple comparison test was used to determine the presence of differences between $2^{-\Delta\Delta Ct}$ values in normoxia and hypoxia treatments for each of the ECM genes examined. Student's t-tests were then used to determine if there were any significant differences between treatment means at the 0.05 level. SPSS version 13.0 for Windows was used to perform the statistical analysis.

6.3 Results and Discussion

This study examined the effects of several different culture conditions on their ability to maintain chondrocyte phenotype. The study compared differences between hypoxic and normoxic conditions in each culture condition as well as differences between each of the culture conditions. The results showed that HDMC culture plays a very important role in the preservation of the chondrocytic phenotype. This study also confirmed that hypoxia is an essential consideration in development of the ideal culture conditions and that there is a synergy between HDMC culture and hypoxia that results in more complete preservation of the chondrocyte phenotype when compared to FICs.

6.3.1 ECM Gene Expression - qPCR

Gene expression profiles for many of the major cartilage ECM genes (collagens I, II, VI, IX, XI and aggrecan) were analyzed to identify those genes which were being differentially regulated between normoxic and hypoxic growth conditions.

6.3.1.1 Collagens Involved in Fibril Assembly (Collagens II, IX, and XI)

6.3.1.1.1 Collagen II

The most important differences in gene expression levels were observed in collagen types II, IX and XI, which are the collagens involved with fibril assembly. Figure 6.2a shows that only HDMCs maintained under hypoxic conditions displayed levels of gene expression for collagen type II which were not significantly different from the levels seen in FICs ($p = 0.14$) Another important observation was that in all culture conditions, chondrocytes grown in hypoxia displayed significantly elevated levels of collagen II gene expression when compared to cells grown in normoxia. For example, the level of collagen II gene expression in HDMCs, AEC and P0 cultures maintained under hypoxic conditions was 8.3, 2.2 and 94 fold higher respectively, than their normoxic counterparts. An interesting finding pertaining to AEC culture conditions was that P0 chondrocytes maintained under hypoxia alone yielded collagen II levels that were higher than both of the AEC treatments. Taken together, these findings demonstrate that: 1) hypoxia plays an essential role in the expression of collagen type II, 2) AEC culture represents an inferior technique for the maintenance of collagen II expression and 3) the synergy between hypoxia and HDMC culture can preserve the levels of collagen II expression seen in FICs.

6.3.1.1.2 Collagen IX

Figure 6.2b shows that the pattern of collagen type IX gene expression levels between normoxic and hypoxic culture conditions was similar to that seen in collagen II gene expression, albeit far more pronounced. HDMCs in hypoxia yielded the highest levels of collagen IX expression of any treatment, even exceeding the levels seen in FICs by 2.8 fold. Given that these HDMCs were

actively producing large amounts of *de novo* ECM, it is not surprising to see levels of collagen IX expression that surpass the levels seen in chondrocytes obtained from fully mature AC. According to Eyre, the amounts of collagen IX can be up to 10 fold higher in developing AC when compared to mature AC(33).

The collagen IX gene expression in HDMCs in hypoxic conditions was a dramatic 10,035 fold higher than P0 cells in normoxic conditions (Fig. 6.2b). This finding underscored the incredible speed and extent of the dedifferentiation process in unpassaged chondrocytes. The fact that all other cultured chondrocytes in this study demonstrated down regulation of this important ECM component so readily may bring into question the findings of previous studies where cultured chondrocytes were used. For example, AECs have long been used to promote and preserve the chondrocytic phenotype. However, when collagen IX gene expression levels were analyzed, AECs faired very poorly relative to HDMCs in hypoxia. Although both groups of AECs expressed higher levels of collagen IX transcripts than P0 cells in normoxic conditions, the HDMCs had strikingly higher levels with a 140.5 fold increase over the AECs in hypoxic conditions and an 878.1 fold increase over the AECs in normoxic conditions. This suggests that HDMC culture conditions satisfy a basic chondrocytic requirement that is not provided by AEC culture. Taken together, these observations demonstrate that the synergy between HDMC growth conditions and hypoxia yield a more natural growth environment that is capable of preventing the down-regulation of collagen IX expression observed in all other treatment conditions.

To date, one of the most important factors in the retention of the chondrocytic phenotype in cultured chondrocytes has been the presence of hypoxia. However, HDMC culture may be considered as important as hypoxia. For example, HDMCs in normoxia yielded levels of collagen II and IX gene expression that were comparable to the levels seen in P0 chondrocytes in hypoxia. This suggests that the benefits of HDMC culture alone are as profound on the preservation of the chondrocytic phenotype as hypoxia.

6.3.1.1.3 Collagen XI

Figure 6.2c shows that hypoxic growth conditions again yielded levels of collagen XI gene expression that were well above the levels seen in the normoxic counterparts. The differences in expression levels between the HDMCs in hypoxic conditions and all other treatments were less pronounced. For example, the HDMCs in hypoxic conditions were only 5.1 fold higher than the HDMCs in normoxic conditions and only 8.0 fold higher than P0 chondrocytes in normoxic conditions.

Interestingly, there was no significant difference in the gene expression levels of collagen XI between the conventional AECs in normoxic conditions (which has been used to redifferentiate chondrocytes) and P0 chondrocytes in normoxic conditions ($p = 0.588$). This suggest that there was no effect from either the 3D alginate environment or TGF-β in the AEC cultures on the levels of collagen XI gene expression relative to P0 chondrocytes. Furthermore, the AECs under normoxia have significantly less collagen XI gene expression than FICs. Given that collagen XI plays a central role in fibril formation, this represents a fundamental deficiency of AEC culture in retaining the chondrocytic phenotype.

The data obtained from collagens II, IX, and XI clearly demonstrate that HDMCs grown under hypoxia express all of the major collagens transcripts necessary for proper fibril formation and display gene expression levels at or above the levels seen in FICs.

6.3.1.2 Collagen I

While appropriate expression of collagens involved in fibril assembly is undoubtedly an important criterion for maintaining a differentiated chondrocyte phenotype, it is equally important to prevent the expression of other aberrant collagens such as collagen type I, which is not normally expressed in healthy AC. With respect to collagen I gene expression, Fig. 6.2d shows that both normoxic and hypoxic conditions for HDMCs yielded levels that were far lower than the

levels observed in any other culture conditions. Moreover, when compared to the levels of collagen I gene expression in FICs, the normoxic and hypoxic cultured HDMCs yielded reductions of 3.6 fold and 6.7 fold respectively (Fig. 6.2d) whereas all other culture conditions produced increased levels of collagen I expression. This observation suggests that the formation of ECM under HDMC conditions alone is sufficient to mitigate the propensity of liberated chondrocytes to upregulate collagen I.

This study confirmed the findings of Murphy and Sambanis (2001) which showed that AEC culture results in a reduction in collagen I expression relative to monolayer culture(34). Importantly, the current findings demonstrate that while redifferentiation procedures involving alginate culture conditions can improve chondrocytic phenotype by decreasing collagen I gene expression relative to standard monolayer culture, the levels of collagen I remain 2.8 fold higher than in FICs and 18.6 fold higher than in HDMCs cultured in hypoxia. These findings further demonstrate the benefits of HDMC culture in maintaining the chondrocytic phenotype but, also provide evidence that elements of the *de novo* ECM are involved in the regulation of collagen I expression.

6.3.1.3 Collagen VI

Figure 6.2e shows that collagen VI gene expression seemed to respond to the various treatment conditions in a manner very similar to collagen I with respect to the different oxygen tensions. Collagen VI, believed to play a central role in the pericellular environment of chondrocytes(35) was expressed at the highest levels in GM38 fibroblasts. As with collagen I, normoxic culture conditions in non-AEC cultures consistently yielded higher levels of gene expression for collagen VI relative to hypoxic counterparts. HDMCs in normoxic conditions and P0 chondrocytes in normoxic conditions both displayed higher levels of collagen VI than FICs (Fig. 6.2e). In contrast, all the hypoxic treatment groups (P0, AEC and HDMC) showed collagen VI gene expression levels that were not significantly different from the levels seen in the FICs ($p = 0.736$, 0.238 and 0.078

respectively). Since all three hypoxic treatments yielded expression levels for collagen VI that were comparable, at least statistically to the levels in FIC, it would appear that hypoxia may play an important role in maintaining low levels of collagen VI in articular chondrocytes. Because collagen VI is expressed at very high levels in fibroblasts and because chondrocytes under normoxia significantly up-regulate collagen VI expression, elevated expression may be associated with chondrocyte dedifferentiation as seen with collagen I. Unlike collagen II, IX and XI which are present throughout the ECM, collagen VI is restricted to the pericellular regions and hence would be expected to be expressed at lower levels under physiologic conditions. As a result, the low levels of collagen VI expression observed in HDMCs maintained under hypoxia may be advantageous for the retention of the chondrocytic phenotype. If confirmed, the beneficial effects of HDMC culture and hypoxia will extend beyond the fibril forming and (FACIT) collagens to also include the mircofibrillar form of collagen.

6.3.1.4 Aggrecan

In addition to promoting cartilage-appropriate collagen expression, the ideal culture conditions should also promote appropriate levels of aggrecan expression. Figure 6.2f shows that chondrocytes maintained under hypoxic growth conditions consistently displayed higher levels of aggrecan gene expression than cells maintained under normoxic conditions, irrespective of the treatment conditions. In particular, HDMCs in hypoxic conditions yielded the highest level of aggrecan expression of any treatment group, even exceeding the levels seen in AECs in hypoxic conditions ($p = 0.005$) and no significant difference ($p = 0.327$) with the level seen in FICs.

Overall, these observations clearly demonstrate that HDMC culture in hypoxia is the best of those conditions studied for the production of the different elements of the cartilage matrix and produce results very similar to FICs. Furthermore, the effects of these conditions on the maintenance of the chondrocytic phenotype

extend beyond simply the expression of collagen genes and involve the non-collagneous components of the matrix as well.

6.3.2 ECM Biosynthesis

After approximately 4 weeks of culturing, a thin, semi-opaque, white layer of material could be observed adhering to the surface of the culture dishes in the HDMC cultures. This layer grew over time and gave rise to the tissue pellets shown in figure 6.3. The cells grown under hypoxic conditions yielded a pellet with a homogeneous, translucent, white appearance, while the cells grown under normoxic conditions yielded a pellet with an opaque, pale yellow appearance (Fig. 6.3). The color change between the pellets maintained under different O_2 tensions provided the first evidence that there might also be differences in their composition. The mass (wet weight) of *de novo* tissue formed by normal articular chondrocytes, which were maintained for 8 weeks under hypoxia (5% O_2) was 2.5 fold higher than the mass of material obtained from chondrocytes maintained under normoxia (20% O_2) (Fig. 6.4). This observation supported the findings of Grimshaw and Mason who found a 10% increase in pellet volumes of chondrocyte cultures maintained for only 7 days under hypoxic conditions when compared to normoxic controls (36).

While the gene expression results provided evidence in support of the differences in *de novo* tissue volumes between HDMCs maintained under normoxic and hypoxic growth conditions, quantitative biochemical analysis of collagen and GAG content was used to confirm them in the ECM. The *de novo* tissue that developed in the HDMC culture provided a unique opportunity to directly compare the amounts of different constituent components that were developed under different oxygen tensions. Figure 6.5 shows that there was a 1.3 fold increase in total collagen in the HDMCs in hypoxic conditions (0.1367 ± 0.003 ug of collagen/ug of DNA) relative to their normoxic counterparts (0.1081 ± 0.001 ug collagen/ug DNA). A larger 6.9 fold increase in GAGs production was observed in the HDMCs in hypoxic conditions (1.771 ± 0.023 ug GAG/ug DNA)

compared to normoxic conditions (0.258 ± 0.031 ug GAGs/ug DNA). Both of these differences were statistically significant ($p < 0.001$). This hypoxia-related increase in GAG production is consistent with a study by Murphy and Polak in which they showed an increase in GAG biosynthesis (1.6 fold) in AECs in hypoxic conditions relative to AECs in normoxic conditions (37). Although the HDMC findings in the present study were based on articular chondrocytes from a single normal donor, Katopodi *et al*., using more readily available tissue from a panel of OA donors, reported that the increase in GAGs appears to be a widespread response of chondrocytes to hypoxic culture conditions(38). Taken together these observations demonstrate that the profound increase seen at the level of gene expression in hypoxic HDMC culture are reflected in increases in the production of ECM molecules such as collagen and GAGs.

While the increase in gene expression levels for aggrecan between normoxic and hypoxic culture conditions was consistent with the increase in GAGs between the treatments, elevated proteoglycan production alone is likely not the only reason for the 590% increase in GAG content observed in the HDMCs maintained under hypoxic conditions. An increase in GAG retention of this magnitude would likely also require a similar increase in collagen to prevent them being lost to the medium. Based on the ratio of collagen to sulfated GAGs in normal AC, a 30% increase in total collagen content in the HDMCs maintained under hypoxic conditions, while statistically significant, likely does not represent an increase of sufficient magnitude to accomplish this. One explanation which could account for the differential retention of GAGs is the differences in relative amounts of collagen II to collagen I in the *de novo* ECMs. Although the assay used can not distinguish between collagen types, when the ratios of gene expression of collagen II to collagen I were compared between these treatments (Fig. 6.6), there was 15.6 fold more collagen II than collagen I gene expression in the HDMCs maintained under hypoxia compared to the HDMCs maintained under normoxia. An increase in the relative abundance of collagen II in the *de novo* ECM could permit increased anchorage of aggrecan and hence the necessary enhanced

retention of GAGs to explain these results. This explanation would account for the greatly elevated GAG retention while simultaneously allowing for the total collagen level in the ECM to be increased to a much lesser extent.

6.3.3 Hypoxia and Matrix Attachment Genes - qPCR

In an attempt to understand the apparent synergistic relationship between hypoxia and HDMC growth conditions in promoting appropriate expression levels of various ECM genes, the level of gene expression of *HIF*-1α and its down stream target (*SOX-9*) were examined in conjunction with three members of the family of ECM attachment and signaling molecules known as integrins.

6.3.3.1 HIF-1α

Surprisingly, the results of this study have shown that the gene expression of *HIF*-1α was higher in normoxic conditions compared with hypoxic conditions in all equivalent culture conditions (Fig. 6.7a). *HIF-1α* gene expression under normoxic conditions was 4.7 fold higher in HDMC, 4.3 fold higher in AECs and 2.5 fold higher in P0 chondrocytes than in their hypoxic counterparts. This was unexpected as several reports have shown that *HIF-1α* gene expression is up-regulated under hypoxic conditions(7,39,40). Interestingly however; *HIF-2α* showed the same response to the different oxygen tensions as *HIF-1α* (data not shown) thus supporting the *HIF-1α* observation. Regardless, it is worth noting the similarity in expression levels between FICs and HDMCs maintained under hypoxia, as these appear to be optimal for the maintenance of the chondrocytic phenotype. While these observations are in sharp contrast to studies that have utilized epiphyseal chondrocytes(7,39,40), Bruker *et al*. stressed that it is unknown whether *HIF-1α* is regulated in the same manner in hypoxic tissues such as articular cartilage(41). Furthermore, it is unclear what role varying oxygen tensions have on HIF regulation in articular chondrocytes. Our observations suggest that not only can *HIF-1α* can be differentially expressed in

articular chondrocytes but it may also be up-regulated by normoxic conditions relative to 5% O_2.

6.3.3.2 SOX-9

Previous work has shown *SOX-9* to be transcriptionally activated by HIF-1α (2) and that it appears to be involved in the expression of a variety of ECM genes during chondrogenesis (13). It was therefore not surprising to find, that in HDMC and P0 cultures the hypoxic environments yielded higher levels of gene expression for *SOX-9* than normoxic counterparts (Fig. 6.7b). Interestingly, the levels of *SOX-9* expression in all 3 treatment groups under hypoxia were not significantly different from the levels observed in the FICs. This observation suggests that 5% oxygen alone may be sufficient to result in the elevation of *SOX-9* levels. Since all hypoxic treatments have similar levels of SOX-9, the differences in downstream ECM gene expression may therefore be directly attributable to the different pericellular environments experienced in the different treatment conditions.

6.3.3.3 Integrins (β1, α1, α2)

Integrins mediate cell-ECM attachment and also provide a mechanism for intracellular signaling. Accordingly, they represent a family of candidate genes which should be examined when exploring connections between changes in the ECM environment and alterations in ECM gene expression in articular chondrocytes. Integrins function as heterodimers consisting of α and β subunits and, in chondrocytes, two of the important herterodimers (α1β1 and α2β1) share a common β -subunit. This study examined the expression of these important anchorage molecules in relation to the different extracellular environments.

In general terms, the most obvious similarity between the gene expression levels of these three integrins was that hypoxic culture conditions produced a decrease in expression when compared to normoxic counterparts in all cases (Fig. 6.7c-e).

However, there were also some important differences in the gene expression levels dependant on the different culture conditions.

6.3.3.3.1 Integrin β1

Figure 6.7c shows that the culture conditions of HDMC in hypoxia generated gene expression levels closest to those seen with the FIC cells. Furthermore, the expression of integrin β1 across the various treatments very closely resembled the profile of expression seen in *HIF-1α* which suggests yet another link between integrin expression and hypoxia.

6.3.3.3.2 Integrin α1

The difference in *α1* gene expression in AECs maintained in normoxia versus hypoxia failed to attain statistical significance ($p = 0.388$) whereas there was a significant difference in the other two culture conditions (Fig. 6.7c). This demonstrates that AEC culture adversely affects the hypoxia-related suppression of integrin α1 common to all other treatments in all of the integrins. Although the relationship between integrin signaling and hypoxia-related gene expression in articular chondrocytes has not been elucidated, it is conceivable that some aspect of AEC culture is interfering with normal cross-talk between the two processes.

6.3.3.3.3 Integrin α2

The expression levels in HDMCs maintained under hypoxic conditions were significantly lower than FICs for both integrin α1 and integrin α2, suggesting that perhaps neither of these cartilage-associated integrins is involved in the HDMC-related maintenance of the chondrocytic phenotype. Since these integrins are known to bind to collagen I, they may both may represent integrins involved in anchorage under normoxic conditions.

Interestingly, integrin α2 gene expression did not appear to be influenced by time in culture, cell density, TGF-β, alginate embedding or the presence of considerable amounts of *de novo* ECM (Fig. 6.7d). In fact, the sole determining

factor in the expression level of integrin α2 appeared to be the oxygen tension under which the chondrocytes were maintained. The emergence of two distinct levels of expression among such a diverse array of treatment conditions suggests a dichotomous (on/off) oxygen-mediated regulatory mechanism. Surprisingly, the FICs expressed levels of integrin α2 were remarkably similar to the levels in all other groups that were in normoxic conditions. While this observation was unexpected, it is not unexplainable, because it could have arisen through oxygen-mediated up-regulation during the 6 hr collagenase digest that was performed under normoxic conditions.

6.3.4 Integrins and Dedifferentiation

The loss of the round cellular morphology has long been associated with chondrocyte dedifferentiation However; it remains unclear whether this is an initiating factor in the dedifferentiation process or a product of it. It may not actually be the round morphology *per se* which is important for inhibition collagen I expression and hence maintenance of the chondrocytic phenotype, but rather the proper matrix attachment and/or subsequent intracellular signaling which leads to the round morphology. This view is supported by a study which concluded that cell-based AC repair strategies will only truly succeed when chondrocytes are provided with scaffolds that yield the appropriate extracellular signals(42). In the case of HDMCs, we believe that the combination of high density culture conditions and sufficient time have enabled the chondrocytes to generate their own appropriate "scaffold". While the current study has not identified an integrin which is induced under hypoxic conditions, it is conceivable that one will be identified which will also bind collagen II and hence provide the appropriate extracellular signals. As a result further study into the role of integrins in maintaining the chondrocytic phenotype in culture is required.

6.3.5 Benefits of 3D Culture

When taken together, these findings shed light on the deficiencies in chondrocyte culture-based studies as well as provide novel insight into the factors that are

currently undermining AC repair strategies. Alginate-encapsulation in normoxic conditions has long been the standard for promoting chondrocytic gene expression, however; the results of our current study clearly demonstrate that HDMC culture in hypoxic conditions is superior at maintaining the gene expression levels of collagen II, collagen IX, collagen XI, aggrecan and *SOX-9* while simultaneously inhibiting up-regulation of collagen I gene expression. When AECs were maintained under hypoxic conditions, there was an enhancement in the level of expression of collagens II (2.2 fold), IX (6.4 fold) and XI (2.8 fold) as well as *SOX-9* (4.1 fold) when compared to conventional AEC culture in normoxic conditions. Interestingly, in P0 cultured chondrocytes the relative hypoxia-associated increases in expression for collagen II (94 fold) and IX(311 fold) were significantly higher, thus indicating that there is an attenuation of the beneficial effects of hypoxia on the expression of these essential ECM genes in AEC culture. Perhaps due to this attenuation the levels of expression of the collagen genes remained well below the levels seen in HDMCs in hypoxic conditions and FICs. While our findings agree with a study by Haudenschild *et al.* which showed that AECs expressed higher levels of aggrecan and collagen II than conventionally cultured chondrocytes(43), our study also confirmed and extended the findings of Darling and Athanasiou which showed that the level of gene expression for aggrecan and collagen II in AECs was greatly reduced compared to native chondrocytes(44). This demonstrates that not only is AEC culture incapable of fully restoring the chondrocyte phenotype, it is also incapable of retaining it. To improve upon the marginal benefits of conventional AEC culture in restoring or preserving the chondrocyte phenotype, a recent study by Coyle *et al.* examined the combined effects of AEC and hypoxic culture. Their study demonstrated the beneficial effects of hypoxia over normoxia on the expression of both aggrecan and collagen II in AECs(45). While we have confirmed their findings, we have also shown that HDMC in hypoxic conditions not only yields higher levels of aggrecan and collagen II but also higher levels of collagen IX and collagen XI gene expression.

In addition to the collagen II, IX, and XI results, HDMC culture in hypoxic conditions also proved to be better for limiting collagen I gene expression when compared to alginate embedding and particularly in hypoxic conditions. HDMCs in hypoxic conditions expressed lower levels of collagen I expression than FICs while AECs in both normoxic and hypoxic culture yielded levels of collagen I gene expression that were significantly higher than the levels seen in FICs (2.8 fold and 6.8 fold respectively). While the use of alginate embedding does represent an improvement over the collagen I levels seen in conventionally grown P0 chondrocytes, it is not as effective as HDMC culture in hypoxic condition due to the increase in collagen I gene expression. Recently a study designed to examine the effects of redifferentiating chondrocytes in micromass culture under hypoxic conditions (1.5% O_2) found that after 14 days, collagen I expression remained high while collagen II remained low when compared to FICs(46). Moreover, based on a study by Katopodi *et al.*, it was shown that even innovative, engineered, hyaluronate-based, matrices like Hyalograft when used in conjunction with hypoxia were incapable of significantly suppressing collagen I gene expression and and failed to retain the chondrocytic phenotype *in vitro* (38). Finally, while many studies have focused on shorter time periods for the sake of cost and convenience, our study was designed to provide the chondrocytes ample time to acclimatize and naturally condition their respective environments. A recent study by Eslaminejad *et al.* which demonstrated the benefits of prolonged culture (ie. 8 weeks) on the gene expression of aggrecan and collagen II in AECs(47) suggested that the prolonged culture time in our experiments may be responsible for the magnitude of some of the observed differences.

6.3.6 Summary

The combined use of HDMC culture and hypoxia in this study has led to a series of striking findings which suggest that *in vitro* conditions now exist that can largely prevent the dedifferentiation process and hence avoid the resulting loss of the chondrocytic phenotype typically associated with conventional chondrocyte culturing. Moreover, this study has shown that the combination of hypoxia and

HDMC culture yield gene expression levels in normal human articular chondrocytes that are similar to the levels seen in FICs and which are superior to the levels seen in alginate-embedded cultures. In addition to these changes, if the hypoxia HDMC culture technique were used in conjunction with techniques such as ACI it would make, for the first time, transplantation of a patient's own undifferentiated chondrocytes a possibility. Further study of the synergy between hypoxia and HDMC culture will yield a better understanding of the precise mechanisms underlying this intriguing combination and provide additional insight into the factors underlying phenotypic instability in chondrocytes.

Figures

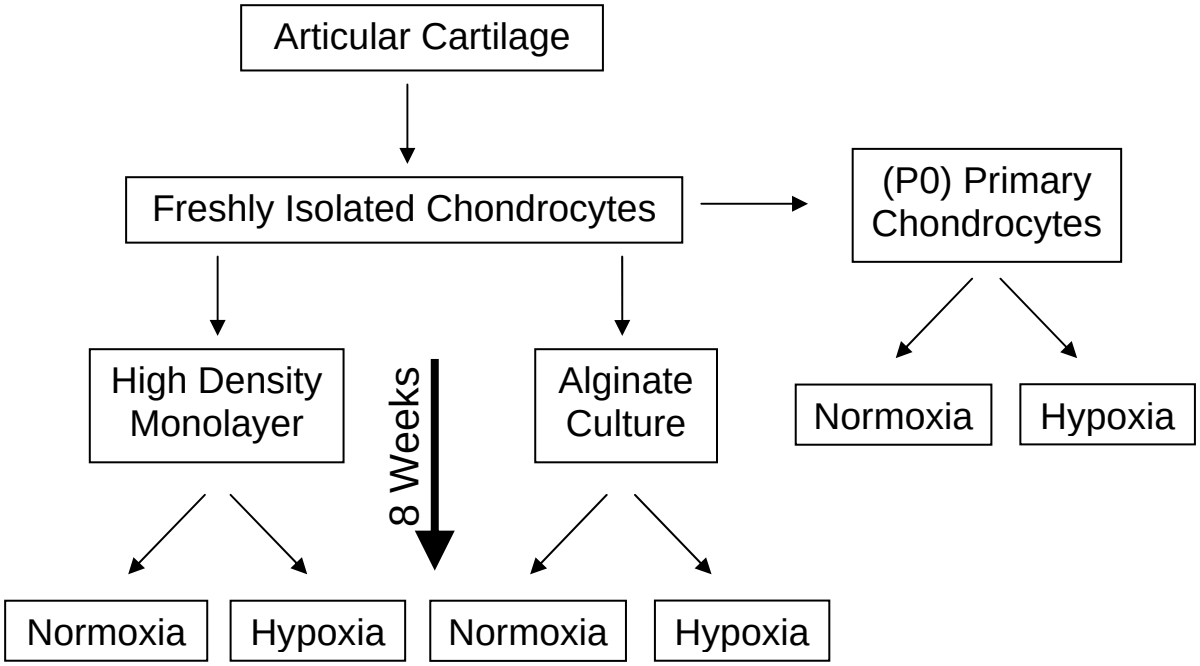

Figure 6.1. Schematic representation of the experimental design showing the three main experimental lineages.

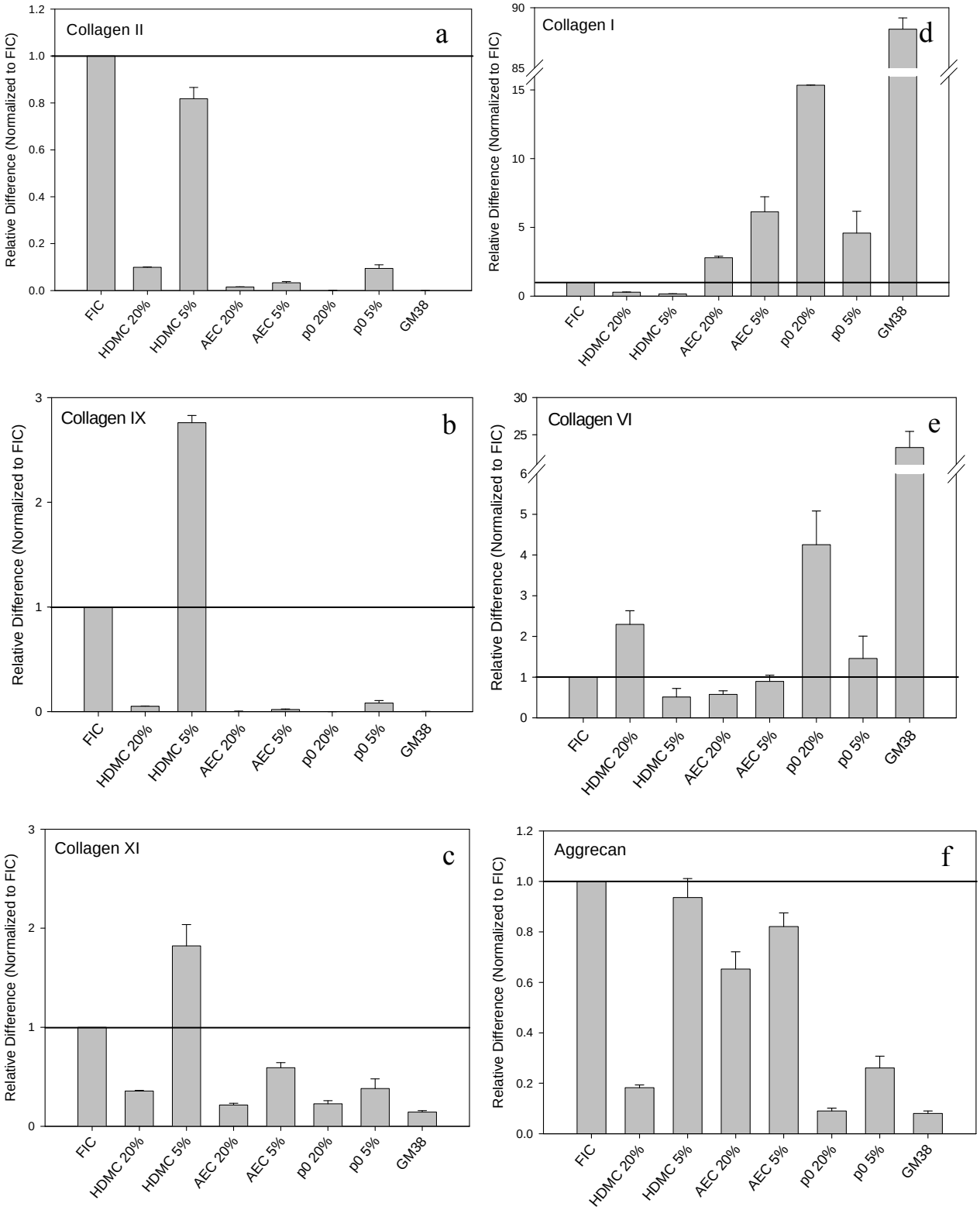

Figure 6.2. Histograms showing the relative fold differences as determined using the ΔΔCt method in normoxia (20% O_2) vs hypoxia (5% O_2) for a) collagen II b) collagen IX c) collagen XI d) collagen I e) collagen VI, f) aggrecan. Values are normalized to GAPDH.

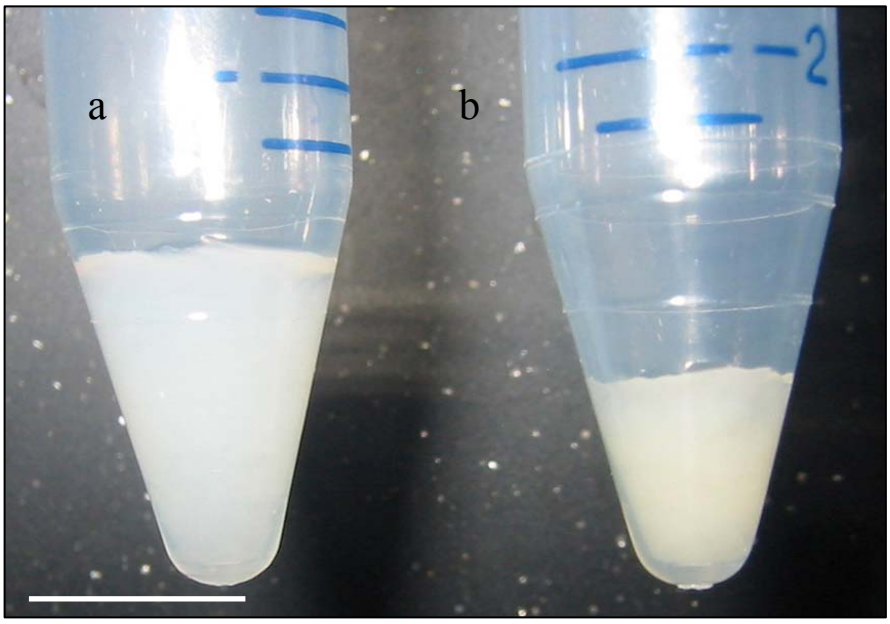

Figure 6.3. Image showing the tissue formed from passage zero chondrocytes after eight weeks in HDMC culture. Cultures were maintained under (a) hypoxic (5% O_2) or (b) normoxic (20% O_2) conditions. Note the difference in the size and color of the pellets. Bar = 1 cm.

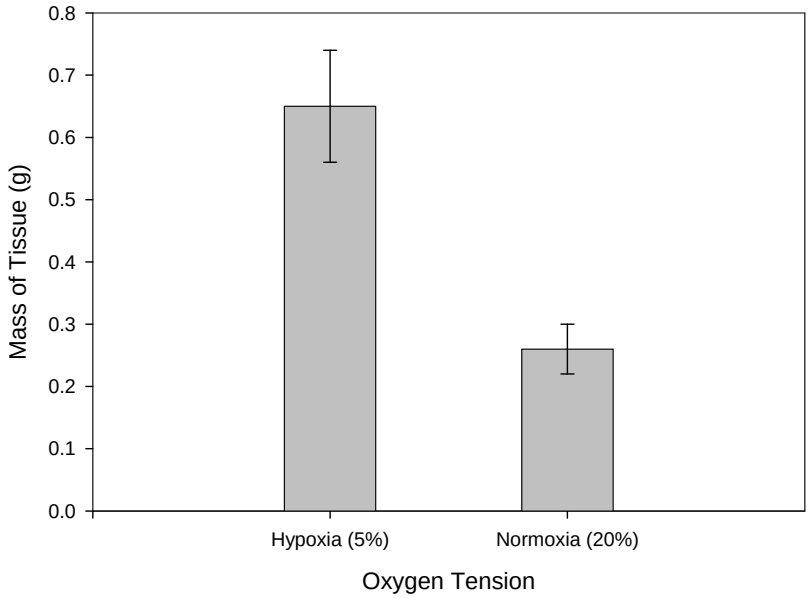

Figure 6.4. Graph showing the mean differences in pellet mass between HDMC cultures maintained under normoxic (20%) and hypoxic (5%) conditions. n = 3

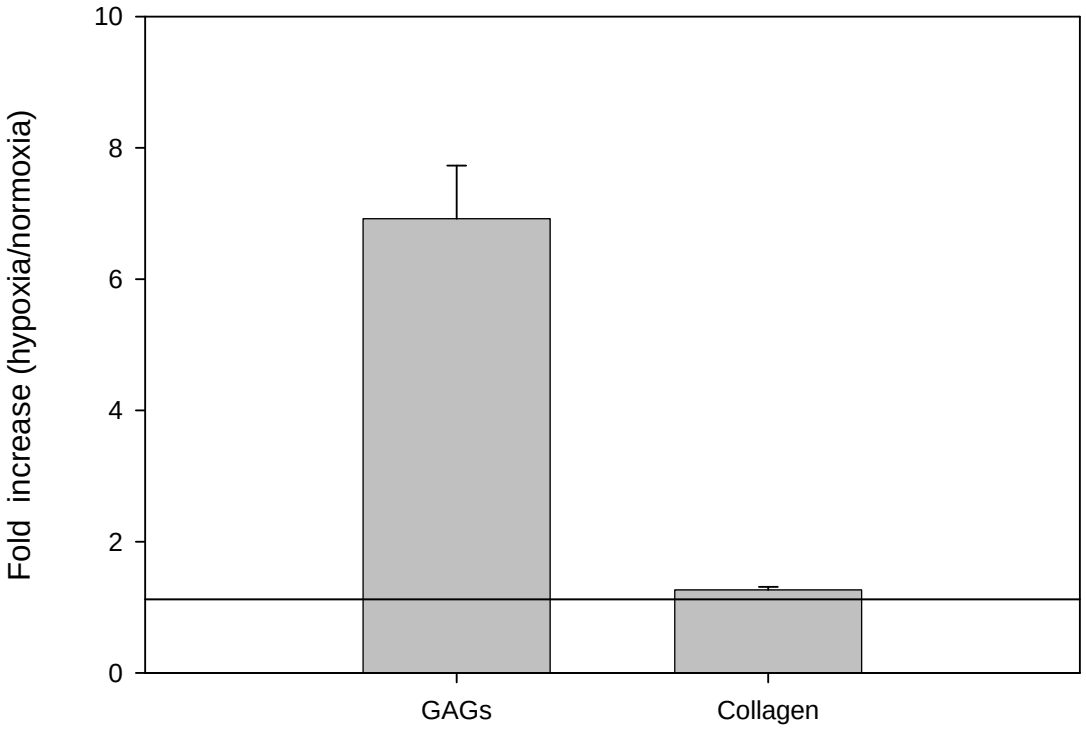

Figure 6.5. Histogram showing the hypoxia-related increase in GAGs and collagens for chondrocytes grown in HDMC culture for 8 weeks. n = 3

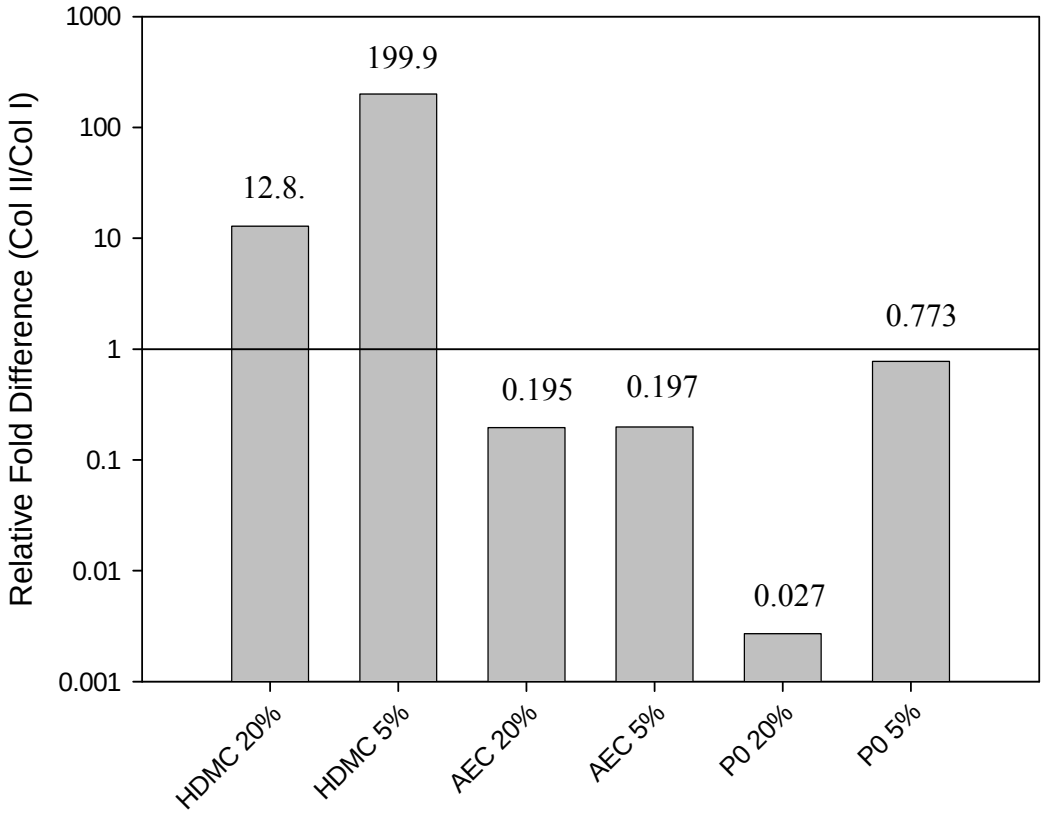

Figure 6.6. Graph showing the ratio of collagen II to collagen I in the HDMC chondrocytes, AECs, and P0 chondrocytes maintained under normoxic and hypoxic conditions. Notice that the ratio of collagen II to collagen I is only above 1 in the HDMC treatments. Also note that alginate embedding of chondrocyte ablates the effect of hypoxia on the balance between collagen I and II expression through a yet undefined mechanism.

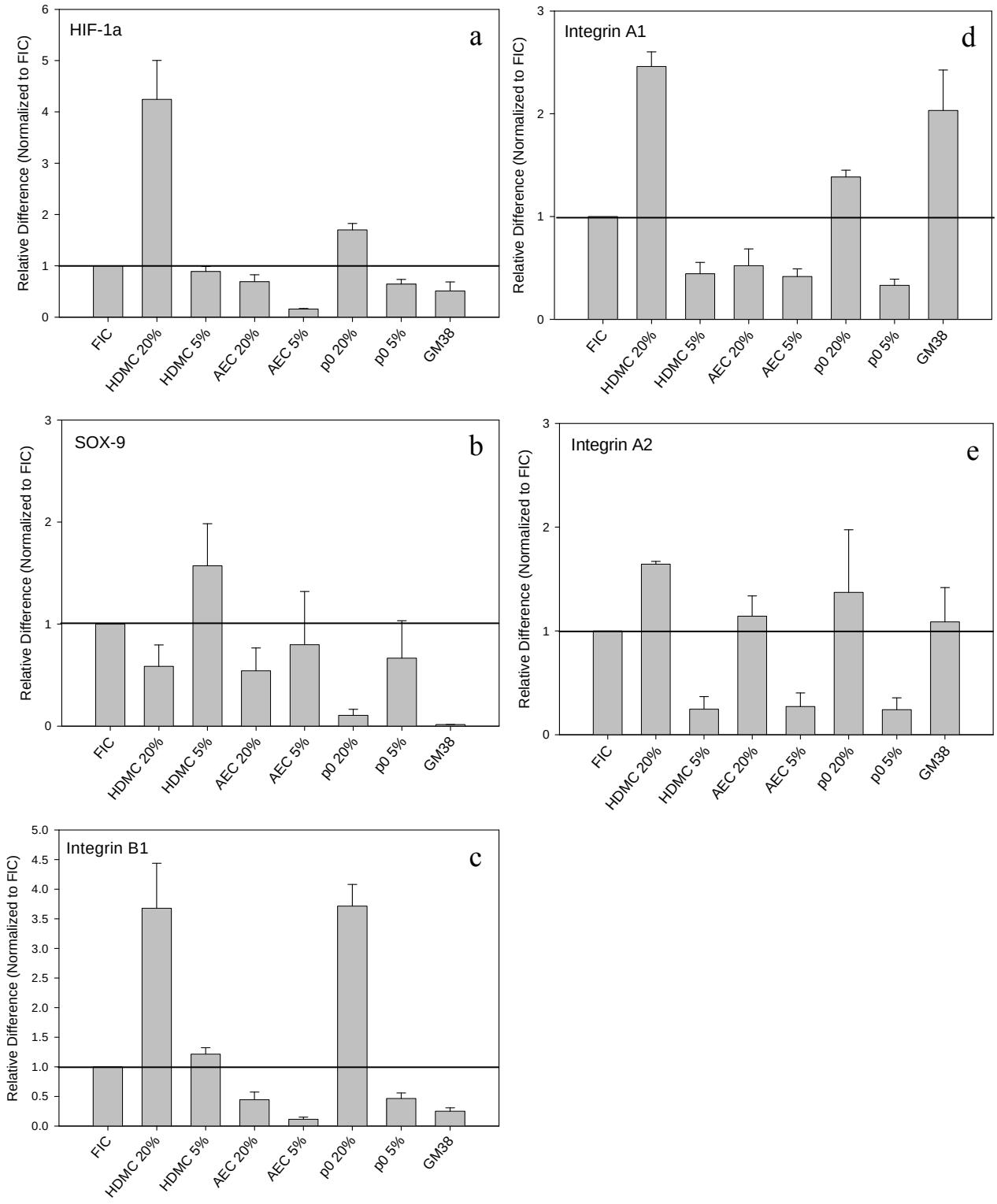

Figure 6.7. Histograms showing the relative fold differences as determined using the ΔΔCt method between cells maintained under normoxia (20% O_2) vs hypoxia (5% O_2) for a) *HIF-1α* b) *SOX-9* c) Integrin β1 d) Integrin α1 e) Integrin α2. Values are normalized to GAPDH.

Table 6.1. Summary of the supplements used in the two media formulations. Note the differences in TGF-β, bFGF and insulin between CGM and CDM.

	TGF-β	bFGF	Insulin	IGF	Transferrin	10% FBS
CGM	-	+	+	+	+	+
CDM	+	-	0.5x	+	+	+

Table 6.2. Summary of RT-PCR primer information showing annealing temperature, forward/reverse sequences and product size.

Gene	°C	Strand	5' - 3" Sequence	bp
Aggrecan	60	F	TCGAGGACAGCGAGGCC	85
		R	TCGAGGGTGTAGCGTGTAGAGA	
Col I	58	F	AGGTGCTGATGGCTCTCCT	105
		R	GGACCACTTTCACCCTTGT	
Col II	58	F	GACAATCTGGCTCCCAAC	257
		R	ACAGTCTTGCCCCACTTAC	
Col VI	58	F	CGTCGATGCCATGGACTTTAT	75
		R	CGGTAGAAGCGGGTCACATAG	
Col IX	56	F	CAGGAAGAGGTCCCAAC	175
		R	GCTGGCTCACAGAAACC	
Col XI	53	F	CGGAGGCAAACATCGTTGAT	104
		R	ATTTGGCTCATTTGTCCCAGAA	
GAPDH	60	F	TGGTATCGTGGAAGGACTCATGAC	189
		R	ATGCCAGTGAGCTTCCCGTTCAGC	
HIF-1α	58	F	CCAGCAGACTCAAATACAAGAACC	121
		R	TGTATGTGGGTAGGAGATGGAGAT	
Integrin β1	58	F	GCCTTACATTAGCACAACACC	263
		R	CATCTCCAGCAAAGTGAAAC	
Integrin α1	58	F	AGAATGCAGCACTCAACTGG	214
		R	TGCAACAAGTACCTCTTCGG	
Integrin α2	60	F	TACGTGCGAGGCAATGACCTA	306
		R	TTTGGGGGTGCAGGATGAAGCT	
SOX-9	58	F	GACTTCCGCGACGTGGAC	186
		R	GTTGGGCGGCAGGTACTG	

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CHAPTER SEVEN: Conclusions

7.1 Healthy Articular Cartilage

Healthy, normal articular cartilage has a homogeneously, smooth, glistening, white, semi-translucent appearance which is typically devoid of any macroscopic imperfections. (Fig. 7.1a). Either as a consequence of aging or the onset of disease, minor changes, such as dulling and softening, occur in the superficial layer of AC, which are indicative of larger changes occurring at the molecular level (Fig. 7.1b). Although it remains unclear, accumulating changes at the molecular level, such as the loss of proteoglycans, may be responsible for the development of minor structural irregularities (Fig. 7.1c). Regardless of their origin, it is the appearance of these structural changes which seem to initiate the process of intrinsic repair observed in this study.

7.2 Intrinsic Repair Model

There appears to be three distinct, consecutive phases of intrinsic articular cartilage (AC) repair in osteoarthritic (OA) cartilage: 1) a damage recognition phase involving an initial collagen I accumulation (Fig. 7.1d), 2) a primary anabolic phase involving the accumulation of aggrecan, collagen II as well as collagen I (Fig. 7.1e) and 3) a secondary anabolic phase involving the *de novo* synthesis of cartilage (Fig. 7.1f).

7.2.1 Collagen I and AC Damage

Based on immunohistochemical staining of OA cartilage, the initial changes in the extracellular matrix (ECM) are apparent in very mildly damaged AC (MG 0-3) and consist of a reduction in aggrecan and collagen II, which is followed by the appearance of collagen I. The presence of collagen I in these AC samples indicates that a damage threshold has been attained within the tissue and that a cellular response is being initiated to deal with it. It is possible that chondrocytes, through their physical attachment and interaction with the ECM are capable of sensing structural disruptions in their immediate surroundings. Resulting alterations occurring either at the plasma membrane (such as the loss of ECM

attachment) or within the cytoskeleton (such as a change in tensional forces), may transduce this information into the nucleus via a series of second messengers where it ultimately results in a corresponding change in gene expression (ie. collagen I).

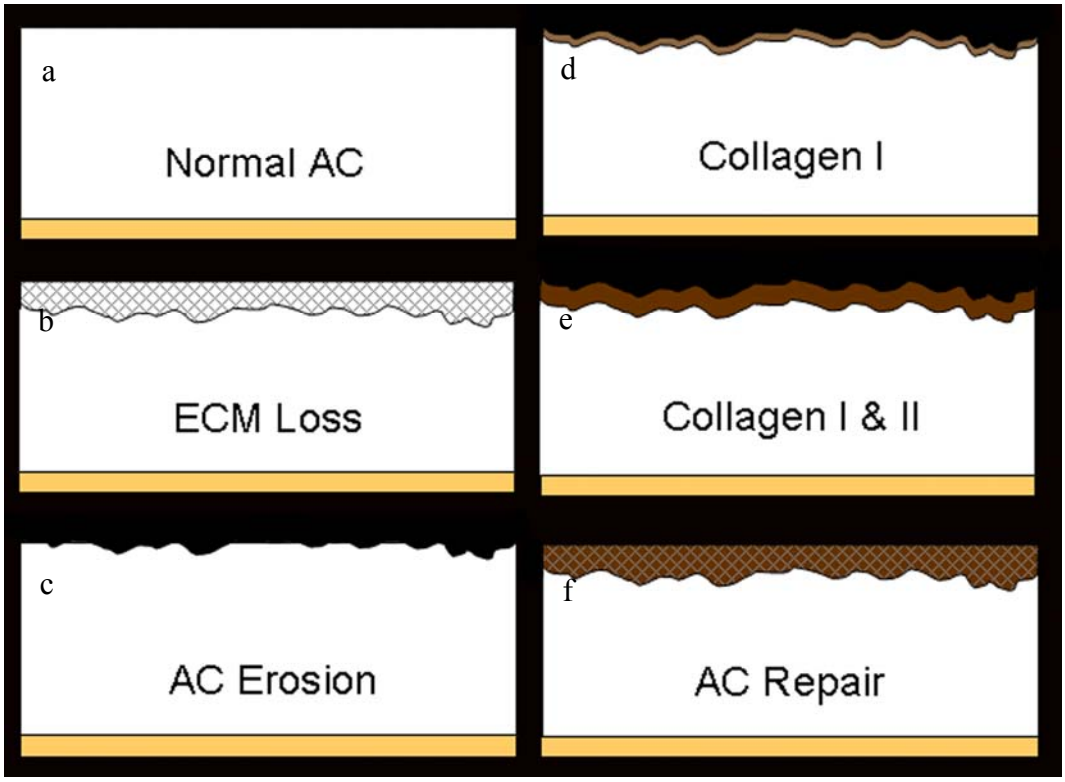

Figure 7.1: Illustration showing the histological progression of AC degeneration and repair in OA. a) Normal intact AC. b) Prior to any signs of overt physical damage, aggrecan and collagen II are lost from the superficial layer. c) Deterioration at the molecular level eventually leads to a loss in structural integrity. d) A damage recognition phase in which collagen I is expressed in association with structurally damaged AC. e) Primary anabolic phase in which collagen I and II are expressed in association with structural damage f) Secondary anabolic phase is which *de novo* synthesis of AC repairs the defect.

7.2.2 Collagen I and AC repair

Given the intense collagen I staining observed in later stages of OA, the accumulation of this collagen in the very early stages of OA likely represents an initial attempt to stabilize the tissue and prevent further damage. Although the role of collagen I in AC remains unclear, it seems reasonable to assume that AC,

like other tissues in the body, would rely on collagen I for tissue stabilization and repair. The role of collagen I in wound healing is well established (1) in contrast to its role in AC. Collagen I is well suited to this role because of its large diameter fibrils which can provide the necessary strength to maintain tissue integrity until repair can be completed.

7.2.3 Alterations in AC metabolism

In the second phase of AC repair, aside from the abundance of collagen I, there is a marked increase in collagen II and aggrecan staining in the superficial-most layers of the remaining tissue. This observation is consistent with reports that show increased expression of aggrecan and collagen II associated with the later stages of OA (2,3). The abundance of aggrecan and collagen II seen in the later stage is in sharp contrast to the absence of these molecules in the initial stage. This net change in AC metabolism may indicate that a second and more serious damage threshold has been attained thus necessitating a more rigorous cellular response. The pronounced net accumulation of chondrocyte-spcecific ECM molecules during this phase may prepare the tissue to enter the final phase of intrinsic AC repair which involves the generation of new cartilage.

7.2.4 Intrinsic Repair in AC

The final stage, which may be dependant on a very narrow range of preexisting conditions, is the *de novo* synthesis of cartilaginous tissue. During this phase, subsets of damaged AC samples demonstrate the ability to generate new cartilage resulting in increased tissue depth. Based on this study, it would appear that full depth repair is a possibility; however, a full range of intermediate repair outcomes likely exist. Unfortunately the repair tissue presented in this study merely represents snapshot in time during the repair process and therefore does not provide any insight into the frequency of such events during the course of OA progression. While research into the metabolic progression of OA is necessary to establish the incidence of these anabolic interludes, the conditions that give rise to the production of *de novo* cartilage could arise multiple times during the course of

disease (Fig. 7.2). Because this study reported the presence of *de novo* fibrocartilage in tissue representing the early stages of OA, while others(4,5) have reported it in the late stages of OA, AC repair likely occurs in a very broad range of damaged AC.

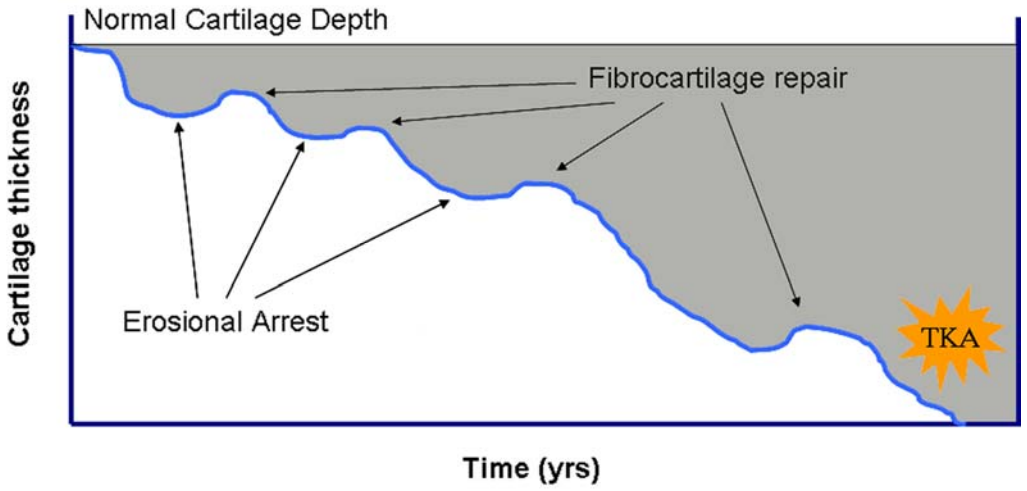

Figure 7.2: A graphical illustration showing the changes in tissue depth as a function of time and metabolic state in a hypothetical OA joint. Anabolic repair events are depicted following periods of erosional arrest in which a change in AC metabolism has occurred. The depth and extent of *de novo* cartilage produced during any repair event could vary considerably.

7.2.5 AC Repair Potential

Although tissue resembling fibrocartilage was observed in this study, one study has actually reported the presence of hyaline-like repair cartilage(6). Since hyaline cartilage is normally devoid of collagen I, it would have been interesting to examine collagen I expression levels in this *de novo* AC. If collagen I is indeed part of an early tissue stabilization and repair response in AC, then it is conceivable that as repair is completed at the molecular level, collagen I may cease to be expressed. During maturation of the repair tissue, according to a recent report, MMP-related remodeling may occur (7), which could remove the collagen I thus giving rise to tissue closely resembling hyaline cartilage.

7.3 Implications of Intrinsic AC Repair

Taken together, these observations suggest that under the right circumstances, even a joint which has previously undergone major physical and enzymatic degradation can synthesize *de novo* AC of considerable quality. This *de novo* synthesis demonstrates that, in the absence of underlying deleterious conditions, chondrocytes themselves are indeed capable of regenerating AC without the benefits of tissue engineering. This *in vivo* finding has significant implications because it precludes many of the assumptions that underlie current tissue engineering approaches. It would now appear that artificial scaffolds (such as hyalograff and PLGA), extrinsic cytokines (such as TGF-β and BMP-7) and even MMP inhibitors are not necessary for the restoration of AC. A better understanding of the factors that permit and promote intrinsic repair tissue should be of considerable therapeutic value in the current effort to treat acute chondral injuries as well as in future endeavors to address OA related cartilage destruction.

7.4 In Vitro Chondrocyte Culture

To generate hyaline cartilage *in vivo*, chondrocytes need to properly control and coordinate the expression levels of numerous ECM genes. However, to understand ECM gene expression and its regulation, non physiologic conditions involving low density 2D monolayer culture have traditionally been used(8,9). These artificial conditions have consistently led to aberrant gene expression patterns and the subsequent loss of the chondrocytic phenotype. Although some recent studies have examined chondrocyte gene expression under physiologic oxygen tensions(10-13), they have continued to overlook the important role played by the extracellular environment.

7.4.1 Chondrocyte Dedifferentiation

When surrounded by the native ECM in AC, chondrocytes display a round morphology. One of the most commonly observed changes in 2D culture associated with the loss of the chondrocytic phenotype is a change in this morphology. Typically, cells grown in monolayer display a stellate, fibroblastic

morphology(14). In an attempt to mimic the cell shape rather than the conditions that yielded it, studies over the past 3 decades have employed a range of 3D culture methods which have only been moderately successful at retaining/restoring an appropriate chondrocytic gene expression pattern.

7.4.2 Anchorage and ECM Signaling

Evidence is now emerging that integrins, through their role in ECM binding may play a very important role in maintaining the chondrocytic phenotype. Egerbacher and Haeusler caution that integrin expression in chondrocytes differs markedly between the *in vivo* environment and the cell culture environment and therefore stress the importance of the genuine matrix milieu(15). The importance of native ECM was supported by the findings of Brodkin *et al.* in which they showed that chondrocytes cultured in 2D on a collagen II matrix alone were unable to retain their normal ECM gene expression profile (16). In a recent study however, Liu *et al.* underscored the importance of native ECM when they discovered that chondrocytes could actually retain their phenotype when only partially digested from their surrounding ECM (17). This suggests that in the case of partial digestion, there were sufficient amounts of native ECM in the immediate pericellular environment (ie. chondron) to provide those chondrocytes with the necessary niche cues to sustain a chondrocyte gene expression profile. According to Heino *et al.* collagen binding integrins, which are known to be involved in anchorage, may play a far more important role in intracellular signaling and downstream ECM gene expression(18). For this reason the *de novo* ECM synthesized in high density monolayer culture was analyzed in the current work for its ability to retain the chondrocytic gene expression profile.

7.4.3 Integrins and Chondrocytes

Integrins are a family of over 20 heterodimeric transmembrane glycoproteins which are composed of an alpha and beta subunit. The extracellular domain of the integrin is responsible for ligand binding, while the intracellular domain interacts with the actin cytoskeleton as well as a host of signaling intermediates. As such,

integrins represent an ideal candidate molecule in chondrocytes that could be responsible for recognizing niche-specific cues in the extracellular environment and subsequently initiating the cytoplasmic signal transduction cascade responsible for activating the appropriate transcriptions factor(s) in the nucleus (Fig. 7.3).

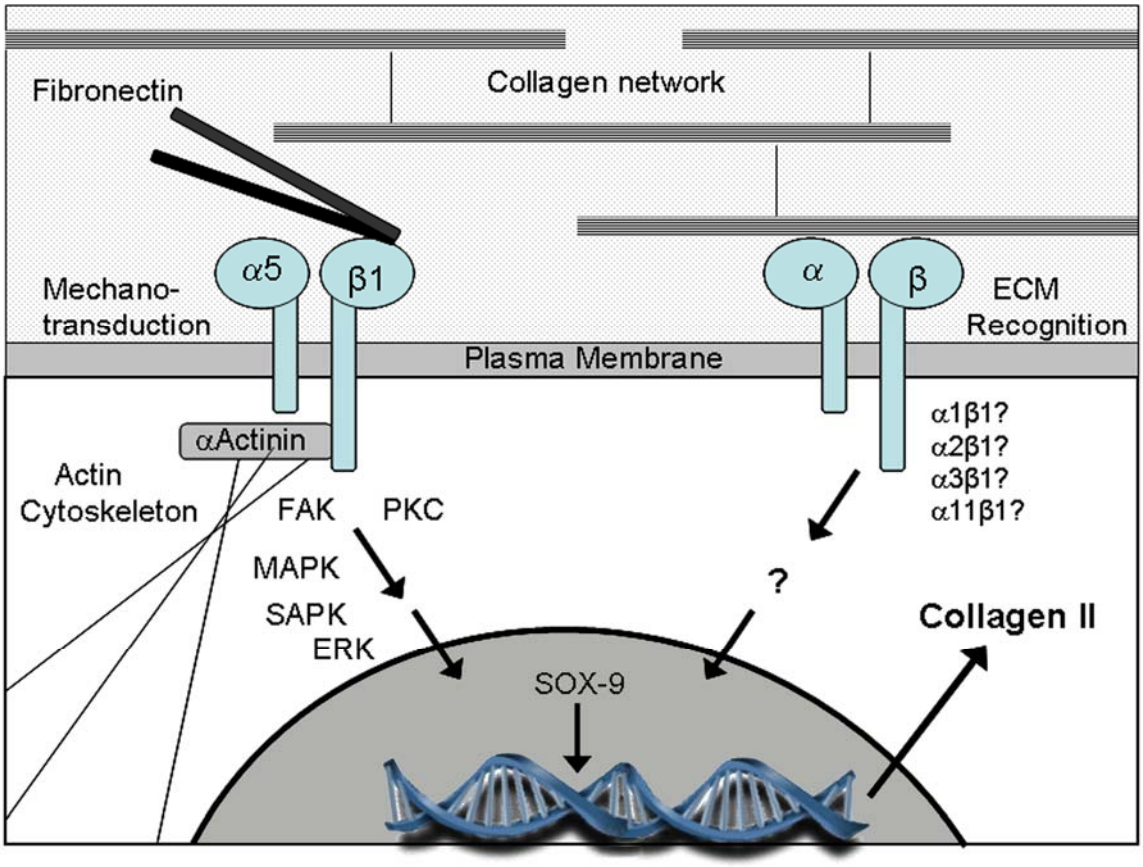

Figure 7.3: Diagram of a chondrocyte illustrating the two different types of integrin-mediated signaling which are capable of modulating collagen gene expression. (Adapted from Millward-Sadler and Salter, 2004)

7.4.4 Integrin Signaling and ECM gene expression

Evidence is emerging that signaling intermediates such as focal adhesion kinase (FAK), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), c-jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and stress-activated protein kinase (SAPK) may all be involved in the integrin-mediated mechanotransduction pathway in chondrocytes (reviewed in 19). In chondrocytes, mechanotransduction is known to rely on the fibronectin-binding

α1β5 integrin. Holmvall *et al.* provided an important initial link between integrin stimulation and ECM gene expression when they demonstrated that α1β5-mediated mechanostranduction results in the rapid upregulation of collagen II (20). Recently while exploring the relationship between mechanotransduction and ECM gene expression, Ng et al. discovered a marked upregulation in *SOX-9* expression following mechanical stimulation (21). Although the precise signaling pathways through which mechanotransduction and niche-cue recognition remain unclear, there is growing evidence that both signals could be mediated by the SOX-9 transciption factor.

7.5 In Vitro Conditions Mimic In Vivo Conditions

The current study took advantage of the innate *in vivo* ability of chondrocytes to regenerate hyaline cartilage and applied it to *ex vivo* chondrocyte culture. In this study, chondrocytes were grown at high density under hypoxic conditions for a prolonged period in order to allow them to appropriately condition their extracellular environment. It was hypothesized that if given an appropriate and important niche cue such as hypoxia, that chondrocytes would eventually express sufficient amounts of collagen II to satisfy their chondrocyte-specific integrin-binding requirements and hence fully recover their chondrocyte phenotype. Although the precise mechanism underlying the restoration of the chondrocytic phenotype in high density monolayer chondrocytes (HDMCs) grown under hypoxia was not extensively examined, the outcome of these experimental conditions confirmed the hypothesis to be correct.

7.5.1 HDMC Culture and ACI

A major problem with all current cell culture models is that they require the initial liberation of chondrocytes form their native ECM. It is plausible that the "damage" caused during liberation results in the disruption of appropriate integrin signaling which is subsequently responsible for the dramatic upregulation in collagen I expression. In the case of HDMCs, the prolonged production of *de novo* ECM under hypoxic conditions may have allowed the collagen II-specific

integrins to be satisfied resulting appropriate intracellular signaling and hence a further reduction in collagen I expression. Regardless of the precise mechanism, the hypoxic HDMC-related retention of the chondrocytic phenotype should have profound implications for tissue engineering approaches such as ACI. When used in place of conventional 2D culture for the amplification of chondrocytes, this new technique will allow the implantation of fully differentiated chondrocytes. The implantation of chondrocytes that display a native ECM gene expression profile rather than dedifferentiated fibroblastic cells expressing low levels of collagen II and high levels of collagen I, should translate into repair cartilage that closely resembles native AC.

7.5.2 In Vitro vs. In Vivo Expression of Collagen I

Based on this work, it is possible that the OA chondrocytes *in vivo* are responding to similar types of mechanical and enzymatic stresses that cultured chondrocytes are subjected to during isolation. It is important to acknowledge that the dedifferentiation process may be compounded by a combination of factors. The initial loss of collagen II-related binding resulting from tissue degradation can initiate a repair response involving the upregulation of collagen I. Prolonged collagen I expression; however, may result in inappropriate niche cues and further loss of chondrocytic phenotype. The signaling that ensues upon integrin binding with the larger diameter collagen I fibrils, may be responsible for subsequent and perhaps more profound alterations in gene expression such as the loss of collagen II expression, which is commonly observed during *in vitro* culture. This has important implication for future treatment modalities and AC-related tissue engineering approaches because if integrins can be prevented from binding to inappropriate niche cues (ie. collagen I), either through the suppression of their expression or through disruption of ligand binding, then a chondrocyte-specific gene expression profile may be retained thus preserving the chondrocyte phenotype.

7.6 Summary

Taken together, this body of work suggests that chondrocytes are capable of producing *de novo* ECM of considerable quality when given the appropriate environmental cues regardless of whether they are *in vivo* or *in vitro*. It also provides a common explanation for the appearance of collagen I in diseased tissue and cultured chondrocytes. Finally, this study also offers insight into the role of integrins as key modulators of phenotype which act at the level of ECM gene expression.

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