The aim of life is self-development. To realize one's nature perfectly, that is what we are here for.

-Oscar Wilde

University of Alberta

THE ROLE OF CULTURED CHONDROCYTES AND MESENCHYMAL STEM CELLS IN THE REPAIR OF ACUTE ARTICULAR CARTILAGE INJURIES

by

Charles Coleman Secretan

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Examining Committee

Dr. Nadr Jomha, Department of Surgery

Dr. Keith Bagnall, Department of Surgery

Dr. Andrew Simmonds, Department of Cell Biology

Dr. John Matyas, Department of Veterinary Medicine, University of Calgary

Mr. Jim Raso, Department of Surgery

Dr. Thomas Churchill, Department of Surgery

This work is dedicated to:

My parents, Glenn and Penny Secretan

If I have seen further it is by standing on the shoulders of giants.
-Isaac Newton

Dr Keith M. Bagnall

For being my mentor and friend

ABSTRACT

Osteoarthritis (OA) is a disease that has significant individual, social, and economic impact worldwide. Although many etiologies lead to the eventual development of OA, one potentially treatable cause is the acute articular cartilage (AC) injury. These injuries are common and have a poor inherent healing capacity, leading to the formation of OA. In an effort to repair AC injuries several treatment strategies have been developed but none have proven completely successful.

Studies examining AC tissue-engineering strategies have suggested that those with the most potential for success involve the introduction of autogenous or allogenous cells to the site of injury. These strategies are designed to encourage creation of a matrix with the appropriate characteristics of normal AC. However, development of a completely successful repair method has proven difficult because the biomechanical properties of normal AC are not easy to replicate, a cell source with the appropriate functional characteristics has not been optimized, and the problem of effective incorporation of a repair construct into the host tissue remains unresolved.

In an effort to more fully understand the cartilage repair process, this work first focused on the development and utilization of an *in vitro* human explant model of AC to study the ability of seeded human chondrocytes to integrate into an AC defect. Further work elucidated the gene expression patterns of cultured adult human chondrocytes and human mesenchymal stem cell (MSC)-derived chondrocytes.

Results from this work determined that cultured human chondrocytes were able to adhere to articular cartilage defects in a viable *in vitro* explant model and produce a matrix containing collagen type II. However, further work with the *in vitro* expanded chondrocytes revealed that these cells have increased expression of collagen type I which promotes the formation of a less durable fibrocartilagenous tissue. This unfavorable expression persisted despite placing the chondrocytes in an environment favoring a chondrocytic phenotype. Further work with MSC-derived chondrocytes demonstrated a similar and unfavorable production of collagen type I. This work represented an important first step towards a treatment for acute AC lesions but it is clear that further work to optimize the culture microenvironment is still required.

Table of Contents

CHAPTER ONE

AN INTRODUCTION TO ACTICULAR CARTIAGE STRUCTURE, FUNCTION, RESPONSE TO INJURY, AND CURENT TREATMENT STRATEGIES

An introduction to articular cartilage structure, function, response to injury and current treatment strategies

1.1 Introduction

Osteoarthritis(OA) is a disease characterized by progressive joint deterioration leading to pain and dysfunction. It is ubiquitous in all populations and has significant individual, social and economic impact worldwide[1-4]. Recently in Canada and in countries around the globe, there has been an increased focus on the distribution of resources with respect to health care. The economic impact of various diseases is being assessed and related to the burden each affliction places on society. OA represents a significant economic burden especially as the population ages. The largest risk factor associated with OA is age[5] and it has been reported by the World Health Organization that 10% of the world's population over the age of 60 years suffers from OA. With the population demographic changing such that in the year 2020 22% of the United States population is expected to be over the age of 65[2], the number of individuals afflicted with OA will continue to increase from 43 to 60 million. In the United States in 2002 it was estimated that the cost of arthritis was as much as 2.5% of the gross national product, with OA being the most common arthropathy contributing to the majority of that amount[3]. This has been projected by others to involve costs of more than 60 billion dollars annually with the expectation that this amount will reach 100 billion dollars by the year 2020[2,3]. In a similar study reported by Badley et al.[6], it was predicted that the number of people in Canada afflicted with arthritis will increase from 2.9 to 6.5 million by the year 2031, an increase of almost 124%, demonstrating that OA will affect our nation in a like

fashion. Disability as a result of OA is also well recognized and it has been observed that OA is second only to ischemic heart disease as a cause of work related disability in males over 50 years of age[3,7]. Although the economic impact of OA is becoming increasingly recognized as outlined above, these statistics do not include the other adverse effects of this disease including individual suffering, pain, psychosocial implications, the effects on other family members and the loss of productivity for society. With the effects of OA on the individual and society, research into the treatments and prevention of this disease is of the utmost importance.

This review will provide an overview of the structure and function of normal articular cartilage and will contrast this to the alterations present in joints affected by OA. The pathogenesis of the osteochondral defect and its relation to the development of OA will receive specific attention, including the current surgical strategies employed in the repair of these defects. Finally, the future of cartilage tissue repair will be discussed with a focus on cartilage tissue engineering.

1.2 Normal Articular Cartilage

Articular cartilage has a complex composition and structure making it remarkably durable and giving it substantial mechanical properties. Lining the load bearing surfaces of diarthrodal joints, articular cartilage provides these joints with a surface possessing a remarkably low coefficient of friction for gliding as well as a resilient load-bearing surface for repetitive motion.

1.2.1 Structure and Composition

Cartilage is a tissue composed primarily of extracellular matrix (ECM) with a relatively sparse population of specialized cells called chondrocytes. It is largely avascular, alymphatic and aneural[8-11]. The chondrocytes make up only 1-3% of the total volume of articular cartilage[8,9,12] and are the only cellular component of articular cartilage[10]. They are mainly anaerobic, have a low metabolic rate under normal conditions, low turnover and communicate predominantly through paracrine mechanisms rather than cell to cell contact[8]. The ECM is composed primarily of collagen, water and proteoglycans with other proteins and glycoproteins making a much smaller contribution. Articular cartilage also has a specialized structural organization which varies throughout its depth. Together the structural organization and composition combine to give articular cartilage its unique mechanical properties[5,8].

In cross section the structure of articular cartilage has been divided into four zones. From superficial to deep these are the superficial zone, the middle or transitional zone, the deep zone and the zone of calcified cartilage. Each of these zones are characterized by the cellular volume and shape, the collagen orientation and size, the fluid dynamics, and proteoglycan content which relate directly to their function[5,8,13]. The lamina splendins is the sheet of fine, densely packed, collagen fibrils covering the superficial zone. It contains little proteoglycans and no cells[10]. Preservation of this layer is crucial for the protection of the deeper layers[8] and also for the regulation of macromolecular traffic within the cartilage[10].

Additionally, the lamina splendins may also be critical in limiting the passage of immunological cells and preserving the immuno-privileged nature of the chondrocytes[10]. The superficial zone contains oblong or flattened cells with their long axis oriented parallel to the cartilage surface. The collagen in this zone is thin, tightly packed and oriented tangential to the surface. The water content is at its highest and the proteoglycan content at is lowest[5]. Type IX collagen in this layer is found interposed between the type II collagen providing resistance to shear[8]. The middle or transitional zone contains cells which are more spherical in shape. The collagen is larger and more randomly organized [5] but still possessing a predominantly oblique orientation[8]. It is hypothesized that this layer provides a transition from the predominantly shear type superficial force to the deeper compressive forces. The deep zone contains chondrocytes which are large, spherical, and organized in a columnar fashion. The collagen is largest in diameter and organized perpendicular to the joint surface. The proteoglycan concentration is at its highest and the water content is at its lowest in this zone[5]. Finally, the zone of calcification is the region of the cartilage which separates the cartilage from the subchondral bone. There are small chondrocytes in this region in a cartilagenous matrix. When stained with hematoxylin and eosin a line termed the tidemark is evident which separates the deep layer of cartilage and the calcified zone[5,8]. This layer orchestrates the complex adhesive process mediating the attachment of the cartilage to the underlying bone. The quantitative structural features possessed by articular cartilage are particularly important if we are to proceed to the engineering of articular cartilage. These quantitative parameters

have now been well defined[12] and knowledge of this will be useful in the understanding of the biomechanical properties of the articular cartilage for repair strategies.

1.2.2 Matrix Composition

The major components of the ECM are collagen, proteoglycans and water. Over 50% of the dry weight of articular cartilage is collagen and of this 90-95% is type II collagen. Other collagens present in smaller amounts within the matrix are types V, VI, IX, X, XI, XII, and XIV[8-10]. The collagens in articular cartilage form a cross-linked network of inter and intramolecular bonds which provide collagen with its 3D stability and contributes to the tensile properties of the tissue.

Proteoglycans are complex molecules containing a protein core with covalently bound polysaccharides[10]. The three major types of glycosaminoglyans in cartilage are chondroitin sulfate, keratan sulfate and dermatan sulfate[5,8]. The larger glycosaminoglyans or aggrecans, including chondroitin and keratin sulfate fill a large volume of the tissue and contribute directly to its mechanical strength while the smaller proteoglycans like dermatin sulfate influence cellular function and may interact with growth factors and cytokines[10]. Each of the sulfate groups on the glycosaminoglycans carries a negative charge, making them highly hydrophilic[5,8].

The affinity of the larger proteoglycans for water is very important. It is generated through two physiochemical mechanisms: first, through Donnan osmotic pressure caused by interstitial freely mobile ions brought in to neutralize the negative charge on the proteoglycans. Second, the entropic nature of

proteoglycans is used to gain volume in solution[5,10]. The turgid nature of the articular cartilage is also contributed to by the repulsive forces which exist between the proteoglycans. Water content and circulation within the tissue is essential for transport of gasses, small molecules, and metabolites[10]. The collagen and proteoglycans together form a strong, solid, matrix which possesses a high affinity for water. The proteoglycans in articular cartilage are not distributed homogeneously but are present in different concentrations at various depths within the matrix. The degree of hydration is determined by a balance of the swelling pressure exerted by the proteoglycans and the constraining forces of the collagen, thereby providing the compressive stiffness of the tissue[5,9].

The homeostatic maintenance of the matrix is orchestrated by the chondrocytes. Even if one were to combine all of the water and macromolecular components in the appropriate ratios, the matrix formed would not function as articular cartilage. The chondrocytes are required to provide the appropriate mechanical properties through organization of the macromolecular framework[10].

1.2.3 Remodeling, Degradative Enzymes, and Growth Factors.

Maintenance of the ECM requires continual turnover of the macromolecular framework and remodeling of the molecular makeup[10]. This occurs in response to alterations in osmolarity, charge density, strain, and the release of mediators. It is orchestrated largely by the chondrocytes[10] and the turnover for the various macromolecular components of articular cartilage varies

considerably. This remodeling is essential for proper cartilage function and preservation[5,9,10,14,15].

Proteolytic enzymes produced by the chondrocytes are involved in the breakdown of the cartilage matrix in normal turnover and in the degradative process. Those thought to be of prime importance are the metalloproteinases (MMPs) (collagenase, gelatinase and stromelysin) and the cathepsins (cathepsins A and B). Each of the MMPs are secreted as proenzymes which are subsequently activated outside the cell by enzymatic modification. In addition, each of these enzymes is inhibited by tissue inhibitor of metalloproteinase (TIMP). The ratio of MMPs to TIMP determines net activity and subsequent matrix turnover [5].

It is also thought that polypeptide growth factors play an important role in the regulation of the synthetic processes of the cartilage matrix. These growth factors act via specific receptor sites on the cell surface[5,11]. The main growth factors studied include platelet-derived growth factor (PDGF), basic fribroblast growth factor (bFGF), transforming growth factor beta (TGF-β), insulin and insulin-like growth factors (IGF-I and IGF-II)[5,13]. It is thought that PDGF elicits a mitogenic effect on chondrocytes. Basic FGF acts on a number of connective tissues, is a potent mitogen, and is also a stimulator of DNA synthesis in adult articular chondrocytes in culture. TGF-β potentiates the effects of DNA synthesis by bFGF, stimulates proteoglycan synthesis and is involved in MSC differentiation to a chondrocyte line[16]. In addition, TGF-β may stimulate the formation of tissue inhibitor of metalloproteinase (TIMP) and plasminogen activator inhibitor–1,2 which are believed to prevent cartilage breakdown. The

proteoglycans decorin, biglycan and fibromodulin regulate TGF-β by sequestering it within the ECM[11]. Finally, IGF-I has been found to stimulate DNA and matrix synthesis in cartilage cultures in vitro[5] and is thought to be the main anabolic growth factor of normal cartilage[11].

Other polypeptides capable of regulating cartilage turnover, the cytokines, include interleukins I-XII, tumor necrosis factor α(TNFα), and interferon (IF)[13,17], the most important of which are IL–1 and TNFα[17]. These cytokines are synthesized by chondrocytes, synovial cells and inflammatory cells. They have a predominantly catabolic effect through the upregulation of the MMPs and act primarily through cell surface receptors. Their expression may be induced by the structural needs of the cartilage or through response to mechanical loading[10]. Their action can be inhibited through receptor antagonists and soluble binding proteins[13].

The maintenance of the ECM and chondrocytic phenotype is not only governed by soluble mediators but also by the interaction of the cells with the ECM. These ECM molecule receptors, called integrins[11], are transmembrane receptors which are known to interact with collagen II and VI, vitronectin, hyaluronin, osteopontin, laminin and fibronectin[11]. These integrins play a pivotal role in the structure and composition of the ECM.

The complex homeostatic process regulating cartilage turnover is still far from being understood and there are several matrix and chondrocytic actions which have yet to be elucidated. What is clear is that within articular cartilage there must be a state of balance between matrix synthesis and matrix degradation

to maintain healthy tissue[17]. As more of the mechanisms are brought to light we may come closer to understanding the roles they play in articular cartilage repair and the pathogenesis of OA. In addition, as solutions to cartilage loss continue to be sought, knowledge of the structural makeup and chondrocyte/matrix interaction will provide a guide as tissue engineering solutions are pursued.

1.3 Osteoarthritis

Osteoarthritis(OA) is a clinical syndrome characterized by retrogressive loss of articular cartilage, accompanied by an attempt, albeit futile, to repair and remodel the lost tissue. It can affect any synovial joint but most commonly affects the joints of the foot, knee, hip, back and hands[5,10,17-22]. In addition to the joint space loss accompanying the destruction of the articular cartilage, pathologic changes in the periarticular region of the joint include subchondral sclerosis, subchondral cysts, osteophyte formation, and varying degrees of synovitis[5,10,17-22]. The other tissues associated with the joint are also affected including the ligaments, joint capsule, metaphyseal bone and muscles. Together all of these factors combine to produce a clinical picture characterized by joint pain, crepitations with joint motion, joint deformity, loss of joint motion and progressive loss of function[5,10,17-22].

1.3.1 Stages of OA

The progression of OA is divided into three overlapping stages based on macroscopic appearance, ECM composition, biochemical alterations and

metabolic activity. The first stage occurs at or just prior to the onset of the presence of fibrillation on the articular cartilage surface. At this point a variety of insults may have occurred which interferes with the ability of the chondrocyte to maintain the ECM. This is characterized by a loss of aggrecan content, glycosaminoglycan chain length and aggregation and a relative maintenance of the overall collagen II content[5,10]. This results in a decreased organization of the macromolecular framework and an increase in the overall water content leading to tissue swelling and increased tissue permeability. These factors combine to decrease the mechanical strength of the articular cartilage and make the tissue more susceptible to subsequent injury[10,23].

The second stage is characterized by a repair response to the initial insult. It appears that the tissue responds appropriately and up regulates both the anabolic and catabolic pathways in an attempt to remodel the tissue. In addition, chondrocyte proliferation[10] is stimulated and collagen II and aggrecan production increased[5]. This produces a characteristic histological appearance of a cluster of chondrocytes surrounding newly synthesized matrix molecules[5,10,23]. There is, however, a net decrease in collagen IX and XI which destabilizes the collagen network and a net decrease in aggrecan resulting from enzymatic degradation in excess of production. These things combine to again decrease the matrix integrity and increase water content[10]. The increased anabolic activity in some instances sufficiently counters the catabolic activity and limits the progression or may even improve the cartilage for a number of years[10]. Unfortunately, this is the exception with continued disease progression predominating.

The third stage of OA is characterized by a progressive loss of articular cartilage and a decrease in the chondrocyte anabolic and proliferative response. This could be due to a decrease in chondrocyte numbers[5,10,18] but also appears to be due to a decreased sensitivity of the chondrocytes to the anabolic growth factors[10]. In either case there is a loss in ability of the existing chondrocytes to maintain the cartilaginous matrix[10,20]. Following the loss of articular cartilage the other features of OA develop including subchondral sclerosis, cyst formation, and osteophyte development.

1.3.2 Etiology

The exact process leading to the development of OA is currently unknown and indeed it is more likely that a myriad of factors cause insult to the joint resulting in the common degenerative endpoint. Osteoarthritis has, however, traditionally been divided into two broad categories based on etiology. The first is primary or idiopathic arthritis. This is the most common form of OA and as the name implies its cause is largely unknown. Idiopathic OA is age associated and its incidence and prevalence rise with increasing age. Secondary OA is less common and develops as a result of joint injury, infection or a number of other hereditary, developmental, neurologic or metabolic insults[5,10,18]. The secondary osteoarthropathies can occur at any time in an individual's life depending on the underlying cause and can affect young adults and in rare instances children.

Having said this, more recently the etiologies of OA have been divided into a new framework. This again involves two broad categories. They are

classified firstly, as **abnormal cartilage stresses**, including trauma, obesity, and other anatomic abnormalities and secondly, **aberrant cartilage physiology** including age, gender, genetic makeup, metabolic factors and inflammation[17]. This newer classification appears to be of use especially when researching treatment of OA and applying various treatment strategies.

Of particular interest in this review are the drivers of abnormal cartilage stress and their effects on the progression of OA. Studies have estimated that between 5% and 20% of young patients suffering traumatic hemarthrosis in the absence of ligamentous injury end up having a chondral defect[8,24] which are often unrecognized[24]. It has been conjectured for some time that large articular cartilage defects (>1cm) result in increased loading of the adjacent cartilage and subsequent progressive deterioration leading to OA[5,20,25]. These lesions are thought to progress in girth and length such that large regions of full thickness cartilage are destroyed[25]. Evidence of continued cartilage breakdown following acute joint injury has been established in a study which analyzed synovial fluid following ACL tear or meniscal injury[10,17,26]. This study, utilizing biologic markers of cartilage deterioration, demonstrated that proteoglycan fragments were elevated two to three fold, MMP levels were increased, cleavage fragments of collagen II elevated and aggrecans in the joint fluid were increased. Interestingly these levels were noted to be high initially but also remained elevated for years following the injury[10,17,26] likely indicating continued cartilage breakdown. This was further substantiated by recent studies which have provided additional insight into cartilage pathophysiology following joint injury. Gelber et al.[27] followed

1321 medical students, 141 of whom suffered joint injury, for a mean time of 36 years. They determined that those suffering joint injuries had a significantly increased risk of developing OA in the injured joint and that individuals in this population should be a target for early intervention. More recently, a Swedish study examined 103 female soccer players who sustained an anterior cruciate ligament (ACL) injury. This study followed these patients for twelve years and determined that those experiencing ACL injuries had a high prevalence of radiographic OA and associated joint pain[28]. This demonstrates that acute joint injury creates an environment that results in altered cartilage metabolism for a number of years. Both abnormal cartilage loading and altered cartilage physiology are likely contributors

OA is the result of a complex series of events precipitated by numerous factors. One of particular interest in this review is the articular cartilage defect. These lesions are common following joint injuries and abnormal mechanical loading on the tissue following these injuries could cause chronic joint pathology leading to OA. Early intervention and treatment of these articular cartilage injuries could prevent the progressive cascade leading to future disability from OA.

1.4 Articular Cartilage Injuries

The exact incidence of chondral injuries is poorly defined. Studies have estimated the incidence of chondral injury to be between 5% and 20% in young patients suffering traumatic hemarthrosis in the absence of ligamentous injury[8,24].

As discussed above, it is likely that large lesions (>1cm^2) of this type contribute to the progression of OA. Much effort has gone into repair of these injuries in an attempt to relieve patient symptoms and prevent the development of OA. The following section will outline some basic yet important aspects of healing and those which apply specifically to articular cartilage. The current surgical techniques utilized to repair chondral defects will also be discussed.

1.5 Repair of Chondral Injuries

1.5.1 Basic Science of Healing

When a vascularized tissue is injured in the body, repair of this tissue is initiated by hemorrhage and formation of a fibrin clot. The injured cells and platelet release mediators which promote the migration of inflammatory cells and undifferentiated cells from the blood to the site of injury. These undifferentiated mesenchymal cells then multiply, differentiate and begin to synthesize extracellular matrix to promote tissue regeneration. These mesenchymal cells are capable of differentiating into a variety of connective tissue cells and, as a result, repair of vascularized tissues is more effective than the repair of unvascularized tissues[5].

1.5.2 Healing of Articular Cartilage Lesions

The above process highlights many of the difficulties with repair that articular cartilage encounters following injuries. It has long been taught that articular cartilage lacks undifferentiated cells, the cells present cannot migrate

within the tissue and the tissue lacks vascularization. The only cell contained within articular cartilage is the highly differentiated chondrocyte and although these cells do proliferate quite rapidly as an individual grows, within mature cartilage cell division declines and there are very few if any mitotic figures[5]. Also the cells are encased within lacunae of the dense extracellular matrix and have limited capacity for migration. It is, however, important to note that when cartilage is injured the cells do respond to the insult by proliferating locally, forming clusters and clones within the ECM, and also begin to produce increased amounts of matrix material, particularly proteoglycans[5,8,10,29]. Although it appears that these cells are responding appropriately, it is insufficient to repair the defect because the new clones appear unable to migrate into the defect and the ECM produced does not fill the defect adequately. In addition, the tissue does not possess undifferentiated cells nor does it have vascularity and as a result there are no sources which could provide the necessary mediators and mesenchymal cells that could migrate to, proliferate and synthesize matrix to fill the defect[5,8,29]. More recent evidence has begun to challenge the traditional dogma which states that there are no multipotent precursor cells within adult articular cartilage. Indeed some have commented that there are precursor cells in the superficial zone of articular cartilage and that they play an important role in joint development [30]. It is thought that a population of these cells persists into adulthood. Despite these interesting findings, more work is still required to fully define these cells that, in any case, are unable to elicit a repair response of any significance.

The healing process described above differs significantly from the series of events which occur if there is subchondral bone injury concomitant with the articular cartilage injury[5,8,29]. Because the subchondral bone is vascular, the processes of hemorrhage, clot information and inflammation are initiated. The necessary undifferentiated multipotent mesenchymal cells, growth factors and humeral proteins are released which allow proliferation, differentiation and matrix synthesis from these cells[5,8,25,29]. Within a matter of weeks the undifferentiated mesenchymal cells have assumed the shape of chondrocytes and begin to produce a matrix containing some type II collagen and proteoglycans. The bony portion of the defect begins to fill with immature bone and by six months has restored the level of the subchondral bone with a combination of bone, type I collagen and hyaline cartilage. The cartilaginous portion of the defect in most instances is filled with a combination of hyaline and fibrocartilage which lacks the appropriate mechanical strength, structure and composition[25]. The chondrocytes also lack a zonal stratification as is present in native tissue[25] and the new collagen generated fails to integrate with that on the periphery of the lesion[25]. This formation of a cartilaginous tissue with inferior biomechanical properties leads to an unfortunate deterioration over the subsequent weeks to months. However, in some instances the cartilage generated possesses sufficient biomechanical integrity and is maintained for longer periods with acceptable function[5]. It is important to understand this repair response as it is the rationale behind a number of the current surgical procedures attempting to repair articular cartilage.

1.5.3 Surgical Options

Lesions in articular cartilage lead to alterations in the joint mechanics. As discussed above, the resultant imbalance of forces on the articular surface at the periphery of the lesion is one of the known causes of OA. In an attempt to prevent this deterioration, a variety of different surgical techniques have been developed in an attempt to repair these lesions. These will each be briefly outlined below.

1.5.3.1 Irrigation and debridement

Arthroscopic lavage alone is effective in reducing painful symptoms[31] associated with a chondral injury in the joint, although the biological mechanism responsible for this has yet to be elucidated. It has been hypothesized that the beneficial results observed are a result of the removal of any inflammatory mediators, MMPs and other pain signaling molecules present in the joint fluid[8,25,29,32]. It has also been postulated that it could remove any excess proteoglycan or aggrecan in the superficial cartilagenous compartment, promoting adhesion of repair cells[25]. Regardless of the initial results, the effects are fleeting, may be due to simple placebo effect[33], and no lasting effect or resultant healing has been documented[8,25].

When lavage is combined with debridement additional benefits have been reported but again there exists no scientific evidence which would indicate this as a long term viable option. When faced with a chondral lesion the surgeon often performs these debridement techniques with the intent of smoothing the lesion

and thereby possibly improving the biomechanical forces at the lesion margin[34]. However, experimental evidence demonstrates this is not the case. Chondral lesions in canine models which had the edges of the lesion beveled through debridement actually demonstrated further progression than those left with vertical edges[34]. Other studies have also demonstrated that chondral lesions which undergo debridement actually have increased chondrocyte loss as a result of cellular apoptosis[35,36]. There have been no prospective randomized trials to assess this intervention and results of the retrospective trials are variable at best[25].

1.5.3.2 Marrow Stimulation Techniques

Marrow stimulation techniques rely on the above discussed principle of access to the multipotent mesenchymal cells, growth factors and humeral proteins present in the marrow compartment. Techniques included in this category include abrasion chondroplasty, microfracture, and Pirdie drilling. Use of these techniques results in incomplete filling of the chondral defect with mechanically inferior fibrocartilage which lacks resilience and provides only temporary defect repair[8,25,29,32]. The main differences in these techniques are the methods utilized to access the intravascular space. Abrasion chondroplasty utilizes a burr, Pirdie drilling a 2mm drill, and microfracture an awl. With each technique there are basic science experiments demonstrating similar results, the formation of fibrocartialgenous tissue repair[8,25,29,32]. Again, utilizing these techniques one cannot expect long term tissue repair[25,37] but may, in the short term, provide symptomatic relief[8,25,29,32,38,39].

1.5.3.3 Radiofrequency Ablation

This preliminary technology involves the use of radiofrequency to penetrate the chondral lesion, shrink the fibrillations and smooth irregularities. This technique is time and energy dependent and varying either level, results in increased cell death which may include a full thickness lesion down to subchondral bone[32,40,41]. Indeed, *in vitro* tests demonstrate chondrocyte death even at minimal settings and short time durations[40,41]. Any individual considering the use of radiofrequency chondroplasty in treatment of chondral lesions should have great reservations and proceed with caution.

1.5.3.4 Periosteal Grafting

This technique involves extending the defect through the subchondral bone, then applying the graft at the base of the defect. The periosteum contains multipotent cells with the potential to differentiate and form different types of cartilage and bone[25,29,32]. There is currently debate as to whether the graft should be placed cambial side down or toward the joint. In either case, what is generated is a defect partially filled with fibrocartilage[25,29,32]. Whether this healing is due to cellular proliferation and differentiation from the graft or from access to the components of the vascular marrow cavity (as described above) is of some debate[42]. In effect, this technique may only involve complicated abrasion arthroplasty. Having said this, this technique may have some future potential and

requires more experimental knowledge to fully evaluate the healing mechanism involved.

1.5.3.5 Autologous Osteochondral Mosaicplasty

This technique involves the arthroscopic or open harvest of small osteochondral dowels from the periphery of the patellofemoral joint or the margins of the intercondylar notch and transferring them into osteochondral defects on the weight bearing surface of the joint[43]. Since its first application in 1992[43] several authors have reported positive clinical results treating osteochondral lesions with this technique[43-48].

Recently two prospective randomized trials have also been undertaken to compare autologous chondrocyte implantation (ACI) (see later) and mosaicplasty. Unfortunately, they did however reach contradictory results with Horas et al[49]. demonstrating greater benefit with mosaicplasty while Bentley et al.[50] had improved results with ACI. There were however limitations to both studies with Horas et al.[49] having a low number of enrolled patients and short duration of follow-up, while Bentley et al.[50] deviated from established post operative care and also treated lesions larger(>4cm) than previously indicated in the mosaicplasty group.

One additional possibility contributing to the discrepancy in results could be that the technically demanding nature of mosaicplasty could lead to significant surgeon to surgeon variability. Mosaicplasty requires that the plugs be placed congruently in all dimensions with respect to the articular cartilage surface. A

dowel left at an inappropriate depth or not placed perpendicular to the surface will generate a suboptimal result because of mechanical overloading in the new position[51]. There are also other aspects of the procedure which could lead to variable results. Larger grafts transplanted to a site on the weight bearing surface leads to unavoidable incongruity of the recipient site[43] making those who utilize larger grafts at a disadvantage. In an attempt to overcome this and generate a more congruent joint surface, individuals have utilized multiple smaller grafts to fill these defects. This unfortunately leads to more areas of intervening fibrocartilage formation which has inferior biomechanical properties[43]. There is also speculation as to whether or not there could be significant donor site morbidity in the future in these patients. With the potential for collateral joint damage accompanied by the technical difficulties associated with the generation of congruency it will be interesting to note the long term outcomes of these procedures.

1.5.3.6 Osteochondral Allografts

This process is similar to the technique for the autografts described above but as the name implies the osteochondral tissue is transplanted from another individual. This procedure is performed far less than mosaicplasty but groups utilizing this technique have favorable long term (mean 10 year) results[52-55]. With this procedure the problems with donor site morbidity, the graft size, and contour of the graft are not as limiting. However, the same technical difficulties with performing the procedure exist. Often logistical complications can make this

procedure demanding on both the patient and the surgeon because they must be ready at short notice if a donor becomes available. As with any allograft the survivability of the transplant depends on cell viability. Studies have demonstrated that maximal cell viability (91.2% ± 5.2%) is achieved when the grafts are implanted fresh (within 7 days of harvest). Grafts which are hypothermically stored (2-8°C) demonstrate a significant decrease in cell viability (80.2% ± 17.4%) after 14 days when stored in ringers lactate but when stored in other proprietary medium had improved viability (83.4% ± 9.8%) up to 28 days[56]. General recommendations therefore suggest that even hypothermically stored allografts should be implanted within 14 days of harvest[56]. There are also concerns associated with the risk of pathogen transmission when transplanting these allografts and this combined with the lack of suitable donor tissue has made this technique less prevalent than mosaicplasty.

1.5.3.7 Autologous Chondrocyte Transplantation

ACI was first performed by Brittberg et al. in October of 1987, with the technique being rapidly adopted in several centers in Europe and the United States[57]. This technique involves the arthroscopic harvest of chondrocytes from one area of the joint followed by the multiplication of the cells *in vitro* in monolayer culture. In a second procedure an arthrotomy is performed, a periosteal patch harvested from the proximal tibia is sutured into place over the chondral defect, and the edges are sealed with fibrin glue. The cultured chondrocytes are then injected under the patch. Several authors have reported

promising results with this technique with a high percentage of good or excellent clinical results in most studies (reviewed by Brittberg[57]). However, a closer observation reveals contradictory information with up to 70% receiving little or no benefit. Although ACI is already utilized in a clinical setting, more experimental research is being produced which challenges the basic scientific tenets of this procedure. The basic premise of ACI relies on the containment of the chondrocytes within the defect, allowing for a gradual development and maturation of a hyaline-like cartilage over time[58]. The post operative regimen also calls for early continuous passive motion to aid in the cartilage maturation[58]. Recent work by Driesang et al.[59] has demonstrated that the periosteal flap utilized to cover the defect is universally delaminated with any motion at the operative joint. Additionally, work by Grande et al.[60] has shown that as little as 8% of the repair cell population is composed of the transplanted chondrocytes. They did not conjecture as to the source of the remaining cells, but the most likely source is the multipotent cells of the marrow cavity following intraoperative disruption of the subchondral bone. It is also well known that chondrocytes multiplied in monolayer culture dedifferentiate, losing their chondrocytic phenotype[61-64]. In order to produce sufficient numbers of chondrocytes for ACI several passages are required with the final transplanted tissue possessing a phenotype more akin to a fibroblast than a chondrocyte. ACI was again cast in critical light when Breinan et al.[65] demonstrated that there was no difference in the chondral defect's histological appearance when comparing ACI, periosteal patch alone, and no defect manipulation in a canine model.

It is possible that the relative success of this procedure in clinical studies is merely due to the containment of a multipotent collection of cells present in clot by a partially adherent periosteal flap. To date ACI has not been compared to several of the other treatment modalities in an appropriate prospective double-blind controlled trial and until it is, one may wonder if the procedure is a complicated and very expensive microfracture technique.

More recently multiple groups have been employing a number of matrices to assist in retention of the chondrocytes within the articular cartilage defects[66-70]. This process termed matrix-induced autologous chondrocyte implantation (MACI) improves on ACI in that it is technically less demanding and shortens operative time. To date it has demonstrated patient outcomes similar to ACI with short- and medium-term follow-up but further work and longer term studies are still required[67,69-73].

1.6 Tissue Engineering

As discussed previously, articular cartilage has a limited capacity for self-repair and current treatment options, although demonstrating some patient benefit, are largely inadequate. With the therapeutic options for these types of injuries being limited, much effort is currently being directed toward methods of tissue engineering in an attempt to solve this complex problem.

Current strategies for cartilage tissue engineering involve the implantation of a supportive matrix, supplementation with stimulatory biomolecules and involvement of either autogenous or allogenous cells. The goal of these matrices

would be to provide a scaffold complete with growth factors and signaling molecules which would serve as a conduit for cell delivery. This living biological substitute would provide a basis for *de novo* extracellular matrix formation, resulting in eventual regeneration of the damaged tissue[74-76]. Each of the components essential to an appropriately engineered tissue will form the basis of the subsequent discussion.

1.6.1 Cells

Cell strategies for cartilage tissue engineering include: (1) stimulation of the tissue progenitor cells already present at the site of injury, (2) harvest and culture expansion of cells to augment those at the defect site, and (3) formation of fully formed tissue in vitro which is later transplanted. Cells currently being investigated for this purpose include mesenchymal stem cells, native progenitor cells, embryonic stem cells and mature chondrocytes.

1.6.1.1 Embryonic Stem Cells

Embryonic stem (ES) cells are those harvested from blastocyst and fetal tissue. Their initial isolation from human embryos in 1998[77] elicited much excitement because of their limitless capacity for proliferation and their potential to be differentiated into any tissue cell type. However, there are no reports of this tissue being utilized successfully in musculoskeletal tissue repair (reviewed by Sharma[78]). There are those who have established differentiation toward the chondrogenic and osteogenic lineages in a murine model[79-82] demonstrating their

potential for use in the field of tissue engineering, but much work is still required in order for utilization of these cells more widely. There is also much ethical debate surrounding this type of stem cell and the requirement of human embryonic tissue for harvest.

Although work with these ES cells could yield much knowledge in the fields of clinical therapeutics and organ development, current focus in the area of cartilage tissue engineering is on cell sources which are less controversial, more readily available, and more easily differentiated into a chondrogenic phenotype.

1.6.1.2 Mesenchymal Stem Cells

Since their discovery in the early 1960's mesenchymal stem cells(MSCs) have been harvested from a number of species including human, canine, rat, rabbit, mouse, goat and guinea pig [reviewed by Baksh[83]]. During that time much work has been devoted to the differentiation of these cells into their terminal mesenchymal phenotypes including bone, cartilage, muscle, adipose tissue, and tendon[83,84]. Of particular interest when working with MSCs is the appropriate combination of growth factors, biomechanical, environmental, and cell to cell cues which will provide a suitable functional state and optimal ECM production. Several groups have been engaged recently in the characterization and application of MSCs to articular cartilage engineering[85-95].

Initial techniques utilized to accomplish this differentiation have involved the pellet technique [84,88] and a variety of methods utilizing alginate [89,90,96,97]as a three dimensional scaffold. In an effort to develop three dimensional

environments with improved biomechanical properties and biophysical stimuli, a variety of different constructs including poly(ethylene glycol), silk, agarose, nanofibrous poly(ε-caprolactone) and poly lactic acid blocks[87,91,94,95,98] have been utilized. Individuals have also documented the *in vivo* chondrocytic differentiation of MSCs in a rabbit model utilizing alginate as the matrix[99]. Others, utilizing a rabbit model, have transplanted a construct developed in vitro into a femoral chondral defect with some success[100]. A particularly well done study by Guo et al.[101] documented the repair of a large osteochondral defect in a sheep model. In this work a beta-tricalcium phosphate scaffold seeded with MSCs was utilized that demonstrated the formation of a cartilage-like tissue which remodeled over time. This was compared to defects filled with scaffold only and unfilled defects and a distinct benefit in the experimental group was verified. The microenvironment necessary for differentiation of MSCs into their functional state is an ongoing work and the scaffolds utilized will be of great importance.

Much effort has also been directed towards the appropriate growth factors required to produce a chondrocytic phenotype from MSCs. Growth factors including TGF-β, BMP, IGF, PDGF, EGF, and FGF have been utilized in multiple combinations, sequences, concentrations and durations of exposure and effects on chondrogenensis have been observed[83,84,88,90,96,101-105]. The most common modality for comparison of the cells types generated is RT-PCR. The transcripts utilized to characterize these cells and attempt to quantify the synthesis of ECM components are collagen I, collagen II, aggrecan and sox 9. Probing for

sox 9 has recently begun to gain favor[102] because it correlates not only with transcription, but also with production of components of the cartilage ECM[106]. Sox 9 is the transcription factor for cartilage gene expression and is stimulated by TGF β super family via the SMAD pathway[16]. TGF β is the one ubiquitous factor in chondrocyte differentiation protocols, making knowledge of the SMAD pathway important to the understanding of the chondrogenesis of MSCs.

Bone marrow derived MSCs are particularly attractive for use in cartilage tissue engineering because they represent an easily acquired autologous cell source with excellent proliferative and regenerative capacity[83]. One aspect of mesenchymal stem cell work that is now beginning to gain attention is the heterogeneity of this complex population of cells. It is recognized that the MSC population represents groups of progenitors at different stages of development and indeed groups have been able to isolate sub-populations of precursor cells that possess increased propensity for neuronal differentiation[107,108]. However, to date the multipotent progenitors within the MSC population responsible for the production of fat, bone and cartilage have not been identified[107] but this is sure to play a critical role as we progress towards synthesis of a viable cartilage replacement. As increased understanding is gained surrounding this complex population of cells, the role of MSCs in articular cartilage tissue engineering will continue to expand.

1.6.1.3 Mature Chondrocytes

The use of mature chondrocytes as opposed to un-differentiated MSCs does seem logical because this allows utilization of cells which are already able to produce the necessary components to generate the ECM. However, there are drawbacks to this approach. As was discussed previously in the section on ACI, chondrocytes removed from their native environment and expanded in monolayer culture dedifferentiate and begin to produce type I as opposed to type II collagen[62-64]. In addition there are questions as to whether there will be future donor site morbidity at the surgical site of cartilage harvest or even if the cells removed from a diseased joint are appropriate for reimplantation. This said, groups have demonstrated redifferentiation of mature chondrocytes with increased expression of type II collagen and aggrecan when cells are grown in three dimensional environments, low oxygen tension, and high density cultures[109-115]. Methods like these which attempt to reestablish or preserve chondrocyte phenotype while expanding these cells *in vitro* could prove to have great utility as these cells are applied in the realm of tissue engineering. However, to date no group has been successful with persistent levels of collagen type I being ubiquitously expressed.

1.6.1.4 Native Progenitor Cells

The use of native progenitor cells has also been utilized as a cells source in tissue engineering. As discussed in the section on articular cartilage injuries and repair, the marrow space is a source of multipotent cells which form the basis of

many of the current surgical repair strategies. Groups have attempted to direct native multipotent progenitor cells to migrate to, proliferate and produce repair tissue through encouragement with locally applied growth factors and scaffolds. Kim et al.[116] utilized a chitosan scaffold seeded with TGF β to encourage healing of a cartilage defect though chemotaxis of the marrow derived progenitor cells and subsequent production of ECM. Hunziker et al.[117] identified the synovium as a potential source for chondral progenitor cells. Their work involved treatment of the cartilage surface with chondroitinase AC or trypsin, fibrin clots, and mitogenic growth factors and observing the histological outcomes for up to 48 weeks in a porcine model.

Unfortunately, access to the marrow and other progenitor cells often comes with undesirable effects. Along with the progenitor cells, a myriad of proinflammatory factors and cytokines are released which promote the formation of fibrocartilagenous scar tissue. If these cells are to be utilized to their full potential these inflammatory conditions must also be controlled.

1.6.2 Scaffolds

Biomaterials both natural and synthetic are beginning to play essential roles in the rapidly expanding field of regenerative medicine. These matrices facilitate the delivery of cells and growth factors, direct the formation of new tissue, and provide biomechanical support to the developing tissue[74-76].

1.6.2.1 Scaffold Characteristics

In order to be successful a scaffold or matrix design must possess a set of generic characteristics. The first of these is biocompatibility. The matrix must be inert when placed *in vivo*, avoiding potentially detrimental inflammatory reactions, immune responses, cytotoxic effects or giant cell reactions[78,118-123]. Factors effecting this include chemical, structural and morphologic features as well as sterilization techniques and potentially toxic chemicals utilized in the process[118]. Secondly, cell function within a matrix is regulated by intricate reciprocal interaction of the cell with its surroundings[76]. A scaffold must provide for adequate cell attachment and appropriate cellular interaction. This includes both the substrate for cell anchorage as well as other proteins and bioactive substances which act together to optimize cell delivery, proliferation and retention[76,78,118-124]. Third, a scaffold must be selected which will biodegrade or resorb at a controlled rate. The degradation byproducts must be easily metabolized and lack any cytotoxic side effects[76,118-120,122-125]. Ideally the process of degradation should occur inversely to ECM production, thereby maintaining an appropriate level of structural integrity while allowing the progressive replacement of the scaffold by the chondrocytic ECM[124]. Next, mechanical integrity of the scaffold required must be able to withstand the physiologic stresses placed on the joint surface while the new tissue is being generated. In addition much of the gene expression and paracrine activity of the chondrocyte is controlled by mechanical deformation of the cells. If the scaffold is too stiff this

process will be hindered and cell to cell contact will be affected[12,25,26,117,118,124,126-130]. Fifth, it is important that the structural anisotropy of native cartilage is generated as the scaffold is being replaced. Cartilage possesses a highly organized structure and advanced scaffold designs that are able to replicate the cellular densities, GAG distribution and collagen orientation are being sought. By imitating this zonal organization, it is hoped that more structurally sound tissues can be engineered[78,131,132]. The porosity of the scaffold is another area requiring particular attention when synthesizing cartilage tissue. Because of the avascular nature of native cartilage, it is essential to have a porous network within the cartilage to allow for the acquisition of nutrients and evacuation of waste by the chondrocytes through the process of diffusion. In addition, the porous structure allows for cellular proliferation and ECM production and distribution. The macroporous structure also relates to the hydration of the scaffold, affecting construct's stiffness and mechanical integrity[124,131-136]. Finally, surgical application must be considered when designing a matrix for implantation. Ease in handling, use of minimally invasive techniques when appropriate, polymerization time, and methods of attachment are all important considerations if an experimental scaffold is to be applied in the clinical realm.

1.6.2.2 Biomaterials

Biomaterials utilized for cartilage tissue engineering fall under two broad categories, those which are naturally derived and synthetic biomaterials.

1.6.2.2.1 Naturally Derived Materials

Collagen Matrices. Collagen is a naturally occurring substance in the joint cavity. As such it is suitable for attachment of cells normally found in musculoskeletal tissues and also possesses degradation products which are physiological. Collagen scaffolds have been utilized for over 20 years and the characterization of both stem cells and chondrocytes within this matrix has been well established in animal models including rabbit, sheep, horse and canine models (reviewed in [25,121]). However, criticism of its use as a scaffold has arisen because biocompatibility has rarely been seriously investigated and when viewed more systematically it has been found to be a problem[25]. Despite this, collagen does offer the opportunity to utilize collagen crosslinking techniques to adjust handling properties and has also been shown to have favorable effects on collagen II and aggrecan production (reviewed in [118]). With these favorable effects, it is possible that different structural modifications could improve the biocompatibility of this matrix, making it a useful biomaterial in tissue engineering[25].

Agarose and Alginate. Agarose and alginate are polysaccharides derived from Asian seaweeds and brown algae respectively[25]. Both have been utilized extensively for chondrocyte culture *in vitro* due to their inertness and ability to maintain a uniformly distributed three dimensional cell conformation[121]. These matrix characteristics have proven useful for the study of cell behavior, autoregulatory signaling systems, chondrogenesis, and MSC differentiation[25,121]. Unfortunately when placed *in vivo* both matrices have produced disappointing results. Agarose demonstrates poor biodegradeability in mammalian biological

systems, likely due to a lack of the necessary enzymes to process the plant derived polysaccharides. Alginate, when implanted alone, inhibits spontaneous repair responses[25,137]. Both have also been criticized because of their poor mechanical stability[118] and their propensity to generate extensive foreign body giant cell reactions[25]. When utilized *in vitro* these naturally derived polysaccharides have shown great utility but their other negative characteristics have thus far prevented their use in humans.

Fibrin. Fibrinogen is a natural component of the intravascular space and when injury occurs it appears in the extravascular space in its polymerized form, fibrin. Fibrin comprises a large component of the subsequent clot and acts to facilitate and promote a healing response. Its degradation is self initiated through substitution with cells in the extra-vascular space and the degradation products are physiologic[25]. These characteristics, on initial observation, make it an attractive option for *in vivo* implantation. In fact, fibrin has been utilized as a matrix for the implantation chondrocytes and differentiation of MSCs in several mammalian species including equine, canine, and rat models[25,121]. However, some drawbacks have been encountered. Fibrin on its own lacks any significant mechanical strength and allogenous fibrin has been shown to elicit an immunological response,[25,121] especially when utilized in high concentration fibrin glues. Although fibrin has been utilized in vivo quite extensively, especially in veterinary medicine, its potential to generate a tissue which surpasses the natural healing process is limited[25].

Chitosan. Chitosan is a copolymer of glucosamine and N-acetylglucosamine which forms a hydrogel when cross-linked with chondroitin sulfate[25]. It can be prepared as thermally sensitive carrier material, is injectable, and displays excellent biodegradeability[25,121]. As already highlighted in this review, a group[116] has utilized a chitosan scaffold seeded with TGF β microspheres to promote the *in vivo* differentiation of MSCs with subsequent formation of a hyaline-like tissue. Others[138] have utilized chitosan scaffolds formed from microfiber spinning and studied the effects on chondrocyte bioresponsiveness. Still others[139] have conjugated chitosan with the bioactive RGD moiety, demonstrating excellent cell adhesion and cell proliferation. Work with this scaffold is still in its infancy but early results are promising.

Hyaluronin. Hyaluronin is a physiologic component of articular cartilage and as such displays excellent biocompatibility and biodegradeability. It has been utilized both *in vitro* and *in vivo* as a chondrocyte and MSC carrier with some success[25,121]. Concern has been elicited with respect to the processing methods utilized to form this macromolecule into a scaffold. To generate a construct useful for this purpose hyaluronin must be cross-linked by esterification. This is thought to lead to a scaffold which has compromised biodegradeability with degradation products which lead to chondrolysis[25]. However, pre-clinical and clinical trials utilizing Hyalograft C, a commercially available esterified hyaluronic acid scaffold, have demonstrated some positive outcomes[68-70,73,140]. Additional concerns with respect to batch variations and potential for pathogen transfer have arisen when considering naturally derived scaffold sources. This

has been particularly prevalent when considering materials isolated from bovine sources because of prion induced spongiform encephalopathy[118,121].

1.6.2.2.2 Synthetic Materials

Various materials have been synthesized into three dimensional scaffolds and utilized for cartilage tissue engineering. This field is rapidly progressing and to date a large number of materials have been investigated. This portion of the review will summarize the most prevalent materials and then will focus on recent work with poly(ethylene glycol) (PEG), a substrate receiving much attention recently in the field of cartilage tissue engineering.

Poly(glycolic acid), Poly(lactic acid), and Poly(lactide-*co*-glycolide). This group of poly(α hydroxyl esters) have their origins is the early 1930's and have been utilized *in vivo* as synthetic suture material since 1970[134]. They have adequate biocompatibility and are now utilized in bioresorbable pegs, screws, plates and rods in many areas of orthopaedics. They undergo mass degradation through hydrolysis of the ester bonds and the degradation products, lactic and glycolic acid, are removed through natural biological processes[118,120,134]. PGA was the first to be utilized in cartilage tissue engineering and since its inception has comprised an extensive proportion of the studies reported in the literature(reviewed in [118,120]). PGA has a highly crystalline, hydrophobic structure and possesses a low solubility in organic solvents. PLA is less crystalline and more hydrophobic making it more soluble in organic solvents, have prolonged biodegradeability and possess more mechanical stability[118,120,134]. With the advent

of PLGA it was noted that the physical, mechanical and degradative properties of the polymer could be controlled by varying the co-polymer ratios[118,120,134]. PGA and PLA have been utilized extensively as scaffolds for articular cartilage regeneration and have been shown to support cell growth and chondrocyte function[118,120]. Both have demonstrated successful healing with a hyaline-like tissue *in vivo* but this tissue was noted to be suboptimal with decreased biomechanical strength and poor subchondral bone formation[118,120]. It has also been noted that chondrocytes seeded in PLA demonstrate reduced cell growth and matrix production when compared to PGA seeded scaffolds[120]. This could be due to the decreased diffusion of nutrients noted when using large volumes of PLA polymer[122]. PLGA has been shown to have low mechanical strength[120] and it has been demonstrated that a local drop in pH as a result of release of acidic degradation products causes tissue necrosis and inflammation[141]. Both of these factors have resulted in its limited use in articular cartilage engineering[120].

Poly(propylene fumarate) and Poly(ethylene oxide). PPF is a hydrophobic linear polymer which undergoes degradation via hydrolysis of the ester linkage. It can be utilized to generate hydrogels when combined with PEG or a vinyl monomer and cross-linked chemically or through UV exposure[125]. The result is an injectable, *in situ* polymerizable, biodegradable and non-toxic matrix[120,122] suitable for chondrocyte encapsulation. PEO is another injectable, biodegradable polymer which can be photopolymerized *in situ*. Experiments utilizing this scaffold have demonstrated its use in chondrogenesis when chondrocytes were combined with the hydrogel and photopolymerized

transdermally in mice[142]. Later Elisseef et al.[143] utilized PEO to photoecapsulate bovine chondrocytes along with microspheres containing TGF β and IGF-I. They noted that the matrix was suitable for cellular proliferation and chondrogenesis following a 14 day incubation *in vitro*. Both PPF and PEO hold significant promise for future use in the field of cartilage tissue engineering.

Poly(ethylene glycol). Poly(ethylene glycol) has shown particular promise in the field of tissue engineering because *in vivo* it possesses biological inertness, thermal stability, resistance to enzymatic degradation, and low immunogenicity[119]. The degradation products are removed in the kidneys with PEG molecules <50kDa being readily filtered. It also possesses characteristics which allow ease of use in the laboratory including inertness in most chemical reactions, solubility in water, and quantitative termination with well defined reacting groups[119]. It is through the reacting groups utilized to generate the cross links and variation in PEG molecular weight that the biodegradable properties and porosity can be tailored. Molecules utilized to form these cross-links include polyrotaxane[144], PEO[145], poly(α-hydroxy acid) derivatives[146], poly(vinyl alcohol)[147], and poly(butylene terephthalate)[148]. Each of these constructs demonstrated acceptable mechanical strength with compression and are capable of chondrocyte culture but as of yet have not been compared to assess chondrogenesis. Very few have thus far made the transition from bench top to intrarticular use.

Of particular interest has been the recent application of protein cross-links within a PEG scaffold. Inspired by work by Wang et al.[149], Park et al[150]

incorporated a MMP sensitive peptide network *in situ* under physiologic conditions. Chondrocytes seeded in this matrix demonstrated increased cellular proliferation and production of ECM products than a similar matrix generated with a non-MMP sensitive peptide. The physiochemical characteristics of these matrices have great potential for modification with the possibility of incorporating functional proteins essential to cell adhesion, integrin binding, growth, and proteolytic remodeling. In a similar experiment another group[151] employing a similar, yet less specific technique, utilized alkali treated collagen to facilitate PEG cross-linking. These techniques have tremendous potential for the generation of cellular microenvironments containing the appropriately cloned proteins which would effectively interact with the implanted cells, promote a chondrocytic phenotype and enhance subsequent matrix production.

The mechanical effects of PEG matrices at varying densities of cross linking have also been examined. As mentioned previously, much of the chondrocyte phenotype is directed by mechanical stimuli. As a result, the deformation characteristics and porosity of the matrix utilized to encapsulate chondrocytes and fill a defect will affect the subsequent quality and distribution of ECM production. This has been noted to be true *in vitro* when examining PEG matrices[152-154]. The modulation of the mechanical and porous properties through cross linking will most certainly be important when moving to *in vivo* applications.

1.6.2.3 Growth Factors

Growth factors are readily apparent and essential components of an artificial tissue engineering strategy. A detailed review of these factors will not be presented here but they have been studied quite extensively *in vitro* and their effects well documented (reviewed in[155]). They have however received limited attention *in vivo* but studies utilizing a porcine model have demonstrated the vast differences which exist between the *in vivo* and *in vitro* environments[156]. Indeed, under *in vitro* conditions various factors do appear to have beneficial effects on chondrogenesis[155], but it appears that *in vivo* only those of the TGF β superfamily have proven effective[25].

1.7 Conclusion

OA is a debilitating disease affecting a vast proportion of our society resulting in significant social, economic, and individual hardship. Multiple etiologies have been identified with the osteochondral defect being a potentially treatable cause. Current surgical strategies for repair of these defects are inadequate and generate fibrocartilage with inferior mechanical properties. This has lead to much investigation in the field of cartilage tissue engineering as a potential solution to this difficult problem.

The generation of synthetic tissue is a complex process. Cellular phenotype is directly related to the biophysical and biochemical properties of the surrounding microenvironment and reasonable duplication of these natural environments has proven difficult. Future work must attempt to address *in vivo*

issues associated with immunogenic responses, tissue integration, scale of tissue repair, and use of appropriate models. Much is still yet to be elucidated and undoubtedly this field will be active for many years to come.

1.8 Reference List

1. Reginster,J.Y. The prevalence and burden of arthritis. *Rheumatology. (Oxford)* **41 Supp 1**, 3-6 (2002).

2. Elders,M.J. The increasing impact of arthritis on public health. *J. Rheumatol. Suppl* **60**, 6-8 (2000).

3. Buckwalter,J.A., Saltzman,C. & Brown,T. The impact of osteoarthritis: implications for research. *Clin. Orthop. Relat Res.* S6-15 (2004).

4. Brooks,P.M. Impact of osteoarthritis on individuals and society: how much disability? Social consequences and health economic implications. *Curr. Opin. Rheumatol.* **14**, 573-577 (2002).

5. Buckwalter,J., Simon,S. & Einhorn,T. Orthopedic Basic Science. Biology and Biomechanics of the Musculoskeletal System. 2nd Edition. (2000).

6. Badley,E.M. & Wang,P.P. Arthritis and the aging population: projections of arthritis prevalence in Canada 1991 to 2031. *J. Rheumatol.* **25**, 138-144 (1998).

7. Lawrence,R.C. *et al.* Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum.* **41**, 778-799 (1998).

8. Alford,J.W. & Cole,B.J. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am. J. Sports Med.* **33**, 295-306 (2005).

9. Aigner,T. & Stove,J. Collagens--major component of the physiological cartilage matrix, major target of cartilage degeneration, major tool in cartilage repair. *Adv. Drug Deliv. Rev.* **55**, 1569-1593 (2003).

10. Buckwalter,J.A., Mankin,H.J. & Grodzinsky,A.J. Articular cartilage and osteoarthritis. *Instr. Course Lect.* **54**, 465-480 (2005).

11. van der Kraan,P.M., Buma,P., van Kuppevelt,T. & van den Berg,W.B. Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. *Osteoarthritis. Cartilage.* **10**, 631-637 (2002).

12. Hunziker,E.B., Quinn,T.M. & Hauselmann,H.J. Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis. Cartilage.* **10**, 564-572 (2002).

13. Dijkgraaf,L.C., de Bont,L.G., Boering,G. & Liem,R.S. Normal cartilage structure, biochemistry, and metabolism: a review of the literature. *J. Oral Maxillofac. Surg.* **53**, 924-929 (1995).

14. Eyre,D.R. Collagens and cartilage matrix homeostasis. *Clin. Orthop. Relat Res.* S118-S122 (2004).

15. Gelse,K., Poschl,E. & Aigner,T. Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* **55**, 1531-1546 (2003).

16. Attisano,L. & Wrana,J.L. Signal transduction by the TGF-beta superfamily. *Science* **296**, 1646-1647 (2002).

17. Mandelbaum,B. & Waddell,D. Etiology and pathophysiology of osteoarthritis. *Orthopedics* **28**, s207-s214 (2005).

18. Mahajan,A., Verma,S. & Tandon,V. Osteoarthritis. *J. Assoc. Physicians India* **53**, 634-641 (2005).

19. Baker,C.L., Jr. & Ferguson,C.M. Future treatment of osteoarthritis. *Orthopedics* **28**, s227-s234 (2005).

20. Buckwalter,J.A. & Martin,J.A. Sports and osteoarthritis. *Curr. Opin. Rheumatol.* **16**, 634-639 (2004).

21. Buckwalter,J.A. Sports, joint injury, and posttraumatic osteoarthritis. *J. Orthop. Sports Phys. Ther.* **33**, 578-588 (2003).

22. Garnero,P. & Delmas,P.D. Biomarkers in osteoarthritis. *Curr. Opin. Rheumatol.* **15**, 641-646 (2003).

23. Dijkgraaf,L.C., de Bont,L.G., Boering,G. & Liem,R.S. The structure, biochemistry, and metabolism of osteoarthritic cartilage: a review of the literature. *J. Oral Maxillofac. Surg.* **53**, 1182-1192 (1995).

24. Buckwalter,J.A. Articular cartilage injuries. *Clin. Orthop. Relat Res.* 21-37 (2002).

25. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage.* **10**, 432-463 (2002).

26. Patwari,P. *et al.* In vitro models for investigation of the effects of acute mechanical injury on cartilage. *Clin. Orthop. Relat Res.* S61-S71 (2001).

27. Gelber,A.C. *et al.* Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. *Ann. Intern. Med.* **133**, 321-328 (2000).

28. Lohmander,L.S., Ostenberg,A., Englund,M. & Roos,H. High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. *Arthritis Rheum.* **50**, 3145-3152 (2004).

29. Meyer,U., Buchter,A., Wiesmann,H.P., Joos,U. & Jones,D.B. Current strategies for articular cartilage repair. *Eur. Cell Mater.* **9**, 39-49 (2005).

30. Hunziker,E.B., Kapfinger,E. & Geiss,J. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. *Osteoarthritis. Cartilage.* **15**, 403-413 (2007).

31. Baumgaertner,M.R., Cannon,W.D., Jr., Vittori,J.M., Schmidt,E.S. & Maurer,R.C. Arthroscopic debridement of the arthritic knee. *Clin. Orthop. Relat Res.* 197-202 (1990).

32. Ritchie,P.K. & McCarty,E.C. Surgical management of cartilage defects in athletes. *Clin. Sports Med.* **24**, 163-174 (2005).

33. Moseley,J.B. *et al.* A controlled trial of arthroscopic surgery for osteoarthritis of the knee. *N. Engl. J. Med.* **347**, 81-88 (2002).

34. Rudd,R.G., Visco,D.M., Kincaid,S.A. & Cantwell,H.D. The effects of beveling the margins of articular cartilage defects in immature dogs. *Vet. Surg.* **16**, 378-383 (1987).

35. Tew,S.R., Kwan,A.P., Hann,A., Thomson,B.M. & Archer,C.W. The reactions of articular cartilage to experimental wounding: role of apoptosis. *Arthritis Rheum.* **43**, 215-225 (2000).

36. Archer,C.W., Redman,S., Khan,I., Bishop,J. & Richardson,K. Enhancing tissue integration in cartilage repair procedures. *J Anat.* **209**, 481-493 (2006).

37. Steinwachs,M.R., Guggi,T. & Kreuz,P.C. Marrow stimulation techniques. *Injury* **39 Suppl 1**, S26-S31 (2008).

38. Sledge,S.L. Microfracture techniques in the treatment of osteochondral injuries. *Clin. Sports Med.* **20**, 365-377 (2001).

39. Bert,J.M. Role of abrasion arthroplasty and debridement in the management of osteoarthritis of the knee. *Rheum. Dis. Clin. North Am.* **19**, 725-739 (1993).

40. Caffey,S., McPherson,E., Moore,B., Hedman,T. & Vangsness,C.T., Jr. Effects of radiofrequency energy on human articular cartilage: an analysis of 5 systems. *Am. J. Sports Med.* **33**, 1035-1039 (2005).

41. Vangsness,C.T., Jr. Radiofrequency use on articular cartilage lesions. *Orthop. Clin. North Am.* **36**, 427-431 (2005).

42. Zarnett,R. & Salter,R.B. Periosteal neochondrogenesis for biologically resurfacing joints: its cellular origin. *Can. J. Surg.* **32**, 171-174 (1989).

43. Hangody,L. & Fules,P. Autologous osteochondral mosaicplasty for the treatment of full-thickness defects of weight-bearing joints: ten years of experimental and clinical experience. *J. Bone Joint Surg. Am.* **85-A Suppl 2**, 25-32 (2003).

44. Laprell,H. & Petersen,W. Autologous osteochondral transplantation using the diamond bone-cutting system (DBCS): 6-12 years' follow-up of 35 patients with osteochondral defects at the knee joint. *Arch. Orthop. Trauma Surg.* **121**, 248-253 (2001).

45. Bentley,G. & Minas,T. Treating joint damage in young people. *BMJ* **320**, 1585-1588 (2000).

46. Berlet,G.C., Mascia,A. & Miniaci,A. Treatment of unstable osteochondritis dissecans lesions of the knee using autogenous osteochondral grafts (mosaicplasty). *Arthroscopy* **15**, 312-316 (1999).

47. Marcacci,M., Kon,E., Zaffagnini,S. & Visani,A. Use of autologous grafts for reconstruction of osteochondral defects of the knee. *Orthopedics* **22**, 595-600 (1999).

48. Chow,J.C., Hantes,M.E., Houle,J.B. & Zalavras,C.G. Arthroscopic autogenous osteochondral transplantation for treating knee cartilage defects: a 2- to 5-year follow-up study. *Arthroscopy* **20**, 681-690 (2004).

49. Horas,U., Pelinkovic,D., Herr,G., Aigner,T. & Schnettler,R. Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial. *J. Bone Joint Surg. Am.* **85-A**, 185-192 (2003).

50. Bentley,G. *et al.* A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee. *J. Bone Joint Surg. Br.* **85**, 223-230 (2003).

51. Cain,E.L. & Clancy,W.G. Treatment algorithm for osteochondral injuries of the knee. *Clin. Sports Med.* **20**, 321-342 (2001).

52. Aubin,P.P., Cheah,H.K., Davis,A.M. & Gross,A.E. Long-term followup of fresh femoral osteochondral allografts for posttraumatic knee defects. *Clin. Orthop. Relat Res.* S318-S327 (2001).

53. Mahomed,M.N., Beaver,R.J. & Gross,A.E. The long-term success of fresh, small fragment osteochondral allografts used for intraarticular post-traumatic defects in the knee joint. *Orthopedics* **15**, 1191-1199 (1992).

54. Beaver,R.J. *et al.* Fresh osteochondral allografts for post-traumatic defects in the knee. A survivorship analysis. *J. Bone Joint Surg. Br.* **74**, 105-110 (1992).

55. Meyers,M.H., Akeson,W. & Convery,F.R. Resurfacing of the knee with fresh osteochondral allograft. *J. Bone Joint Surg. Am.* **71**, 704-713 (1989).

56. Ball,S.T. *et al.* The effects of storage on fresh human osteochondral allografts. *Clin. Orthop. Relat Res*. 246-252 (2004).

57. Brittberg,M., Tallheden,T., Sjogren-Jansson,B., Lindahl,A. & Peterson,L. Autologous chondrocytes used for articular cartilage repair: an update. *Clin. Orthop. Relat Res.* S337-S348 (2001).

58. Gillogly,S.D. Treatment of large full-thickness chondral defects of the knee with autologous chondrocyte implantation. *Arthroscopy* **19 Suppl 1**, 147-153 (2003).

59. Driesang,I.M. & Hunziker,E.B. Delamination rates of tissue flaps used in articular cartilage repair. *J. Orthop. Res.* **18**, 909-911 (2000).

60. Grande,D.A., Pitman,M.I., Peterson,L., Menche,D. & Klein,M. The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. *J. Orthop. Res.* **7**, 208-218 (1989).

61. Brodkin,K.R., Garcia,A.J. & Levenston,M.E. Chondrocyte phenotypes on different extracellular matrix monolayers. *Biomaterials* **25**, 5929-5938 (2004).

62. Benya,P.D., Padilla,S.R. & Nimni,M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313-1321 (1978).

63. Benya,P.D., Padilla,S.R. & Nimni,M.E. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. *Biochemistry* **16**, 865-872 (1977).

64. Cheung,H.S., Harvey,W., Benya,P.D. & Nimni,M.E. New collagen markers of 'derepression' synthesized by rabbit articular chondrocytes in culture. *Biochem. Biophys. Res. Commun.* **68**, 1371-1378 (1976).

65. Breinan,H.A. *et al.* Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. *J. Bone Joint Surg. Am.* **79**, 1439-1451 (1997).

66. Jones,C.W. *et al.* Matrix-induced autologous chondrocyte implantation in sheep: objective assessments including confocal arthroscopy. *J. Orthop. Res.* **26**, 292-303 (2008).

67. Behrens,P., Bitter,T., Kurz,B. & Russlies,M. Matrix-associated autologous chondrocyte transplantation/implantation (MACT/MACI)--5-year follow-up. *Knee.* **13**, 194-202 (2006).

68. Tognana,E., Borrione,A., De Luca,C. & Pavesio,A. Hyalograft C: hyaluronan-based scaffolds in tissue-engineered cartilage. *Cells Tissues. Organs* **186**, 97-103 (2007).

69. Nehrer,S. *et al.* Three-year clinical outcome after chondrocyte transplantation using a hyaluronan matrix for cartilage repair. *Eur. J. Radiol.* **57**, 3-8 (2006).

70. Marcacci,M. *et al.* Articular cartilage engineering with Hyalograft C: 3-year clinical results. *Clin. Orthop. Relat Res.* 96-105 (2005).

71. Bartlett,W. *et al.* Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. *J. Bone Joint Surg. Br.* **87**, 640-645 (2005).

72. Manfredini,M., Zerbinati,F., Gildone,A. & Faccini,R. Autologous chondrocyte implantation: a comparison between an open periosteal-covered and an arthroscopic matrix-guided technique. *Acta Orthop. Belg.* **73**, 207-218 (2007).

73. Pavesio,A. *et al.* Hyaluronan-based scaffolds (Hyalograft C) in the treatment of knee cartilage defects: preliminary clinical findings. *Novartis. Found. Symp.* **249**, 203-217 (2003).

74. Langer,R. & Tirrell,D.A. Designing materials for biology and medicine. *Nature* **428**, 487-492 (2004).

75. Griffith,L.G. & Naughton,G. Tissue engineering--current challenges and expanding opportunities. *Science* **295**, 1009-1014 (2002).

76. Lutolf,M.P. & Hubbell,J.A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotechnol.* **23**, 47-55 (2005).

77. Thomson,J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147 (1998).

78. Sharma,B. & Elisseeff,J.H. Engineering structurally organized cartilage and bone tissues. *Ann. Biomed. Eng* **32**, 148-159 (2004).

79. Nakayama,N., Duryea,D., Manoukian,R., Chow,G. & Han,C.Y. Macroscopic cartilage formation with embryonic stem-cell-derived mesodermal progenitor cells. *J. Cell Sci.* **116**, 2015-2028 (2003).

80. Kramer,J., Hegert,C. & Rohwedel,J. In vitro differentiation of mouse ES cells: bone and cartilage. *Methods Enzymol.* **365**, 251-268 (2003).

81. Kawaguchi,J., Mee,P.J. & Smith,A.G. Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. *Bone* **36**, 758-769 (2005).

82. Bronson,S.K. Bone nodule formation via in vitro differentiation of murine embryonic stem cells. *Methods Enzymol.* **365**, 241-251 (2003).

83. Baksh,D., Song,L. & Tuan,R.S. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J. Cell Mol. Med.* **8**, 301-316 (2004).

84. Pittenger,M.F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147 (1999).

85. Cancedda,R., Dozin,B., Giannoni,P. & Quarto,R. Tissue engineering and cell therapy of cartilage and bone. *Matrix Biol.* **22**, 81-91 (2003).

86. Grande,D.A., Mason,J., Light,E. & Dines,D. Stem cells as platforms for delivery of genes to enhance cartilage repair. *J. Bone Joint Surg. Am.* **85-A Suppl 2**, 111-116 (2003).

87. Huang,C.Y., Reuben,P.M., D'Ippolito,G., Schiller,P.C. & Cheung,H.S. Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* **278**, 428-436 (2004).

88. Johnstone,B., Hering,T.M., Caplan,A.I., Goldberg,V.M. & Yoo,J.U. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* **238**, 265-272 (1998).

89. Kavalkovich,K.W., Boynton,R.E., Murphy,J.M. & Barry,F. Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system. *In Vitro Cell Dev. Biol. Anim* **38**, 457-466 (2002).

90. Ma,H.L., Hung,S.C., Lin,S.Y., Chen,Y.L. & Lo,W.H. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. *J. Biomed. Mater. Res. A* **64**, 273-281 (2003).

91. Noth,U., Tuli,R., Osyczka,A.M., Danielson,K.G. & Tuan,R.S. In vitro engineered cartilage constructs produced by press-coating biodegradable polymer with human mesenchymal stem cells. *Tissue Eng* **8**, 131-144 (2002).

92. Sekiya,I., Vuoristo,J.T., Larson,B.L. & Prockop,D.J. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proc. Natl. Acad. Sci. U. S. A* **99**, 4397-4402 (2002).

93. Stock,U.A. & Vacanti,J.P. Tissue engineering: current state and prospects. *Annu. Rev. Med.* **52**, 443-451 (2001).

94. Wang,Y., Kim,U.J., Blasioli,D.J., Kim,H.J. & Kaplan,D.L. In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. *Biomaterials* **26**, 7082-7094 (2005).

95. Williams,C.G. *et al.* In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. *Tissue Eng* **9**, 679-688 (2003).

96. Yang,I.H. *et al.* Comparison of phenotypic characterization between "alginate bead" and "pellet" culture systems as chondrogenic differentiation models for human mesenchymal stem cells. *Yonsei Med. J.* **45**, 891-900 (2004).

97. Steinert,A. *et al.* Chondrogenic differentiation of mesenchymal progenitor cells encapsulated in ultrahigh-viscosity alginate. *J. Orthop. Res.* **21**, 1090-1097 (2003).

98. Li,W.J. *et al.* A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials* **26**, 599-609 (2005).

99. Diduch,D.R., Jordan,L.C., Mierisch,C.M. & Balian,G. Marrow stromal cells embedded in alginate for repair of osteochondral defects. *Arthroscopy* **16**, 571-577 (2000).

100. Lee,J.W., Kim,Y.H., Kim,S.H., Han,S.H. & Hahn,S.B. Chondrogenic differentiation of mesenchymal stem cells and its clinical applications. *Yonsei Med. J.* **45 Suppl**, 41-47 (2004).

101. Guo,X. *et al.* Repair of large articular cartilage defects with implants of autologous mesenchymal stem cells seeded into beta-tricalcium phosphate in a sheep model. *Tissue Eng* **10**, 1818-1829 (2004).

102. Indrawattana,N. *et al.* Growth factor combination for chondrogenic induction from human mesenchymal stem cell. *Biochem. Biophys. Res. Commun.* **320**, 914-919 (2004).

103. Mastrogiacomo,M., Cancedda,R. & Quarto,R. Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells. *Osteoarthritis. Cartilage.* **9 Suppl A**, S36-S40 (2001).

104. Worster,A.A. *et al.* Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. *J. Orthop. Res.* **19**, 738-749 (2001).

105. Wang,W.G., Lou,S.Q., Ju,X.D., Xia,K. & Xia,J.H. In vitro chondrogenesis of human bone marrow-derived mesenchymal progenitor cells in monolayer culture: activation by transfection with TGF-beta2. *Tissue Cell* **35**, 69-77 (2003).

106. Kolettas,E., Muir,H.I., Barrett,J.C. & Hardingham,T.E. Chondrocyte phenotype and cell survival are regulated by culture conditions and by specific cytokines through the expression of Sox-9 transcription factor. *Rheumatology. (Oxford)* **40**, 1146-1156 (2001).

107. Phinney,D.G. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle* **6**, 2884-2889 (2007).

108. Phinney,D.G. & Prockop,D.J. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* **25**, 2896-2902 (2007).

109. Tallheden,T. *et al.* Gene expression during redifferentiation of human articular chondrocytes. *Osteoarthritis. Cartilage.* **12**, 525-535 (2004).

110. Schulze-Tanzil,G. *et al.* Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. *Cell Tissue Res.* **308**, 371-379 (2002).

111. Benya,P.D. & Shaffer,J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**, 215-224 (1982).

112. Francioli,S.E. *et al.* Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: Relevance for Automated Bioreactor Systems. *Tissue Eng* (2007).

113. Domm,C., Schunke,M., Steinhagen,J., Freitag,S. & Kurz,B. Influence of various alginate brands on the redifferentiation of dedifferentiated bovine articular chondrocytes in alginate bead culture under high and low oxygen tension. *Tissue Eng* **10**, 1796-1805 (2004).

114. Domm,C., Schunke,M., Christesen,K. & Kurz,B. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. *Osteoarthritis. Cartilage.* **10**, 13-22 (2002).

115. Schulze-Tanzil,G. *et al.* Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. *Cell Tissue Res.* **308**, 371-379 (2002).

116. Kim,S.E. *et al.* Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: implications for cartilage tissue engineering. *J. Control Release* **91**, 365-374 (2003).

117. Hunziker,E.B. & Rosenberg,L.C. Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane. *J. Bone Joint Surg. Am.* **78**, 721-733 (1996).

118. Woodfield,T.B., Bezemer,J.M., Pieper,J.S., van Blitterswijk,C.A. & Riesle,J. Scaffolds for tissue engineering of cartilage. *Crit Rev. Eukaryot. Gene Expr.* **12**, 209-236 (2002).

119. Tirelli,N., Lutolf,M.P., Napoli,A. & Hubbell,J.A. Poly(ethylene glycol) block copolymers. *J. Biotechnol.* **90**, 3-15 (2002).

120. Lu,L., Zhu,X., Valenzuela,R.G., Currier,B.L. & Yaszemski,M.J. Biodegradable polymer scaffolds for cartilage tissue engineering. *Clin. Orthop. Relat Res.* S251-S270 (2001).

121. Frenkel,S.R. & Di Cesare,P.E. Scaffolds for articular cartilage repair. *Ann. Biomed. Eng* **32**, 26-34 (2004).

122. Elisseeff,J. Injectable cartilage tissue engineering. *Expert. Opin. Biol. Ther.* **4**, 1849-1859 (2004).

123. Temenoff,J.S. & Mikos,A.G. Injectable biodegradable materials for orthopedic tissue engineering. *Biomaterials* **21**, 2405-2412 (2000).

124. Hutmacher,D.W. Scaffold design and fabrication technologies for engineering tissues--state of the art and future perspectives. *J. Biomater. Sci. Polym. Ed* **12**, 107-124 (2001).

125. Drury,J.L. & Mooney,D.J. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **24**, 4337-4351 (2003).

126. Buckwalter,J.A. & Mankin,H.J. Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr. Course Lect.* **47**, 477-486 (1998).

127. Grodzinsky,A.J., Levenston,M.E., Jin,M. & Frank,E.H. Cartilage tissue remodeling in response to mechanical forces. *Annu. Rev. Biomed. Eng* **2**, 691-713 (2000).

128. Guilak,F., Butler,D.L. & Goldstein,S.A. Functional tissue engineering: the role of biomechanics in articular cartilage repair. *Clin. Orthop. Relat Res.* S295-S305 (2001).

129. Hasler,E.M., Herzog,W., Wu,J.Z., Muller,W. & Wyss,U. Articular cartilage biomechanics: theoretical models, material properties, and biosynthetic response. *Crit Rev. Biomed. Eng* **27**, 415-488 (1999).

130. Waldman,S.D. *et al.* Effect of biomechanical conditioning on cartilaginous tissue formation in vitro. *J. Bone Joint Surg. Am.* **85-A Suppl 2**, 101-105 (2003).

131. Sachlos,E. & Czernuszka,J.T. Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. *Eur. Cell Mater.* **5**, 29-39 (2003).

132. Yang,S., Leong,K.F., Du,Z. & Chua,C.K. The design of scaffolds for use in tissue engineering. Part II. Rapid prototyping techniques. *Tissue Eng* **8**, 1-11 (2002).

133. Liu,X. & Ma,P.X. Polymeric scaffolds for bone tissue engineering. *Ann. Biomed. Eng* **32**, 477-486 (2004).

134. Hutmacher,D.W., Goh,J.C. & Teoh,S.H. An introduction to biodegradable materials for tissue engineering applications. *Ann. Acad. Med. Singapore* **30**, 183-191 (2001).

135. Hutmacher,D.W. Scaffolds in tissue engineering bone and cartilage. *Biomaterials* **21**, 2529-2543 (2000).

136. Hutmacher,D.W., Sittinger,M. & Risbud,M.V. Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems. *Trends Biotechnol.* **22**, 354-362 (2004).

137. Mierisch,C.M. *et al.* Chondrocyte transplantation into articular cartilage defects with use of calcium alginate: the fate of the cells. *J. Bone Joint Surg. Am.* **85-A**, 1757-1767 (2003).

138. Subramanian,A., Lin,H.Y., Vu,D. & Larsen,G. Synthesis and evaluation of scaffolds prepared from chitosan fibers for potential use in cartilage tissue engineering. *Biomed. Sci. Instrum.* **40**, 117-122 (2004).

139. Masuko,T. *et al.* Chitosan-RGDSGGC conjugate as a scaffold material for musculoskeletal tissue engineering. *Biomaterials* **26**, 5339-5347 (2005).

140. Marcacci,M. *et al.* Articular cartilage engineering with Hyalograft C: 3-year clinical results. *Clin. Orthop. Relat Res.* 96-105 (2005).

141. Lu,L. *et al.* In vitro and in vivo degradation of porous poly(DL-lactic-co-glycolic acid) foams. *Biomaterials* **21**, 1837-1845 (2000).

142. Sims,C.D. *et al.* Injectable cartilage using polyethylene oxide polymer substrates. *Plast. Reconstr. Surg.* **98**, 843-850 (1996).

143. Elisseeff,J., McIntosh,W., Fu,K., Blunk,B.T. & Langer,R. Controlled-release of IGF-I and TGF-beta1 in a photopolymerizing hydrogel for cartilage tissue engineering. *J. Orthop. Res.* **19**, 1098-1104 (2001).

144. Lee,W.K. *et al.* Novel poly(ethylene glycol) scaffolds crosslinked by hydrolyzable polyrotaxane for cartilage tissue engineering. *J. Biomed. Mater. Res. A* **67**, 1087-1092 (2003).

145. Riley,S.L. *et al.* Formulation of PEG-based hydrogels affects tissue-engineered cartilage construct characteristics. *J. Mater. Sci. Mater. Med.* **12**, 983-990 (2001).

146. Bryant,S.J., Chowdhury,T.T., Lee,D.A., Bader,D.L. & Anseth,K.S. Crosslinking density influences chondrocyte metabolism in dynamically loaded photocrosslinked poly(ethylene glycol) hydrogels. *Ann. Biomed. Eng* **32**, 407-417 (2004).

147. Martens,P.J., Bryant,S.J. & Anseth,K.S. Tailoring the degradation of hydrogels formed from multivinyl poly(ethylene glycol) and poly(vinyl alcohol) macromers for cartilage tissue engineering. *Biomacromolecules.* **4**, 283-292 (2003).

148. Malda,J. *et al.* The effect of PEGT/PBT scaffold architecture on the composition of tissue engineered cartilage. *Biomaterials* **26**, 63-72 (2005).

149. Wang,C., Stewart,R.J. & Kopecek,J. Hybrid hydrogels assembled from synthetic polymers and coiled-coil protein domains. *Nature* **397**, 417-420 (1999).

150. Park,Y., Lutolf,M.P., Hubbell,J.A., Hunziker,E.B. & Wong,M. Bovine primary chondrocyte culture in synthetic matrix metalloproteinase-

sensitive poly(ethylene glycol)-based hydrogels as a scaffold for cartilage repair. *Tissue Eng* **10**, 515-522 (2004).

151. Taguchi,T. *et al.* Encapsulation of chondrocytes in injectable alkali-treated collagen gels prepared using poly(ethylene glycol)-based 4-armed star polymer. *Biomaterials* **26**, 1247-1252 (2005).

152. Bryant,S.J., Anseth,K.S., Lee,D.A. & Bader,D.L. Crosslinking density influences the morphology of chondrocytes photoencapsulated in PEG hydrogels during the application of compressive strain. *J. Orthop. Res.* **22**, 1143-1149 (2004).

153. Bryant,S.J. & Anseth,K.S. Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage. *J. Biomed. Mater. Res. A* **64**, 70-79 (2003).

154. Bryant,S.J. & Anseth,K.S. Hydrogel properties influence ECM production by chondrocytes photoencapsulated in poly(ethylene glycol) hydrogels. *J. Biomed. Mater. Res.* **59**, 63-72 (2002).

155. van den Berg,W.B., van der Kraan,P.M., Scharstuhl,A. & van Beuningen,H.M. Growth factors and cartilage repair. *Clin. Orthop. Relat Res.* S244-S250 (2001).

156. Hunziker,E.B. Growth-factor-induced healing of partial-thickness defects in adult articular cartilage. *Osteoarthritis. Cartilage.* **9**, 22-32 (2001).

CHAPTER TWO

THE EFFECTS OF INTRODUCING CULTURED HUMAN CHONROCYTES INTO A HUMAN ARTICULAR CARITLAGE EXPLANT MODEL

The effects of introducing cultured human chondrocytes into a human articular cartilage explant model.

2.1 Introduction

Articular cartilage (AC) is an avascular tissue with poor innate healing capacity following injury. As a result, articular surface injuries progress such that full-thickness cartilage loss eventually occurs and osteoarthritis (OA) ultimately develops[1-3]. Damage to AC is common[4,5] and the resultant OA causes significant disability that has major social and economic implications[3,6-8]. The significance of these injuries has led to the development of numerous AC repair strategies in an attempt to heal these defects early in the disease process thereby halting the subsequent degeneration[9]. Unfortunately, none of these strategies has proven completely successful and consequently other, more effective methods of AC repair are being developed[1].

Development of a completely successful repair method has proven difficult because the complex structural and biomechanical properties of normal AC are not easy to replicate[10-13]. Studies examining tissue-engineering strategies have suggested that those with the most potential for success involve the introduction of autogenous or allogenous cells to the site of injury either alone or embedded within a supportive scaffold[15-17]. These strategies involve creation of a new matrix with the appropriate characteristics of normal AC which will be expected to be able to incorporate into the adjacent native tissue. However, the problem of effective incorporation of the construct into the host tissue remains a major obstacle[14]. This problem has garnered much attention with focus being placed on an enzymatic preparation of the defect site to encourage cellular

adhesion[14,18] and the prevention of cell death and tissue necrosis in the tissue adjacent to the defect[19]. The region immediately adjacent to an AC defect is of particular concern because the tissue has been shown to become acellular following injury which may inhibit effective host-tissue integration leading to failure of the repair[14,19]. An understanding of this process of cell loss along with the prevention of cellular apoptosis adjacent to regions of injury is limited. For example, it has not been determined whether implanted cells are ever able to become integrated into the acellular regions of matrix[20]. Further study of the processes occurring at the construct–host interface is essential to enhance current repair techniques and generate new approaches to AC repair.

In the current study, an *in vitro* human explant model of AC was utilized to study the ability of seeded human chondrocytes to adhere and integrate into an AC defect. A human model was specifically employed because animal models have many features which differ significantly from the human and which may affect results. For example, animal models display different cell densities, tissue architecture, and biomechanical properties which might contribute to a microenvironment where the healing potential and tissue behavior may differ significantly from that found in human tissue[1]. Additionally, it is well recognized that chondrocyte behavior, matrix production and cellular motility are all influenced extensively through the paracrine signals received from other chondrocytes and matrix interactions[11,20,21]. Therefore, it is important that an appropriate model be used for the study of construct integration and possible matrix repopulation.

Currently there are few studies documenting the results of long term *in vitro* explant studies utilizing human tissue[22]. This study simply involved introducing cultured chondrocytes into a standardized site of injury in human AC explants. The purpose of this study was to assess the ability of the implanted chondrocytes to attach at the site of injury, develop a matrix, and incorporate into the devitalized host matrix adjacent to the defect.

2.2 Methods

2.2.1 Preparation of Cartilage Explants

Samples of AC were obtained from patients undergoing total knee arthroplasty (TKA) for OA. Chamfer trimmings from the weight-bearing portion of the less affected condyles (Outerbridge grade 0-1 [23]) were selected and a total of thirty-six 10mm diameter, cylindrical dowels were harvested. The dowels included full-thickness AC as well as the underlying subchondral bone. The dowels were washed thoroughly with PBS to remove any debris present from harvesting and also to remove detrimental catabolic mediators present in the osteoarthritic joint and then incubated for 48 hours in Chondrocyte Growth Medium (CGM) (Cambrex).

2.2.2 Treatment of the osteochondral dowels

The 36 dowels were divided into three equal groups: 1) uninjured, 2) injured and 3) injured and seeded (Figure 2.1). The dowels in groups 1 and 2 acted as controls while those in group 3 formed the experimental group. A

conical injury that traversed the full-thickness of the AC was generated in the latter two groups using a hand-held countersink bit to minimize any detrimental effects of pressure and friction. A pilot study demonstrated that the technique produced a standard conical injury measuring 4.05±0.64mm in diameter. Dowels were cultured in six-well plates in CGM, incubated at 37ºC and 5% CO_2, and the medium was changed every 3-4 days throughout the experiment. Dowels were harvested at 0, 7, 14 and 28 days.

2.2.3 Chondrocyte Isolation and Labeling

Chondrocytes to be implanted were obtained from fresh, human AC harvested from the distal femur (Human Organ Procurement and Exchange; HOPE program, University of Alberta). Following an 8 hour digestion in Collagenase (Sigma) (1mg/ml) in serum-free DMEM-F12 the chondrocytes were isolated and plated in monolayer on 100mm plastic tissue culture dishes. They were incubated at 37ºC and 5% CO_2 and passaged (1:2) when approaching confluence. Cells were collected at the end of passage 3 and labeled using PKH 26 dye (Sigma) according to the manufacturer's specifications. In this way, cells could be identified following implantation. The labeled cells were divided into $1.0x10^6$ aliquots and pipetted gently into the medium directly overlying the injured portion of the dowels in Group 3 (Figure 2.1) on day 0.

2.2.4 Cell Viability/Cytotoxicity and Cell Incorporation

On the appropriate days, the dowels were removed from the medium and the AC was cut from the subchondral bone using a scalpel. The discs of AC were divided in half which was at the apex of the defect in the injured dowels. One half of the AC disc was serially sectioned at 70µm intervals using a vibratome while the other half was fixed immediately in formalin (4%). The fresh, sectioned tissue was viewed immediately using a Leica fluorescence microscope to document seeded cell incorporation within the defect in the dowels of Group 3 (injured and seeded). Fresh sections from each dowel were also stained for viability with the Live/Dead kit (Invitrogen) following the manufacturers specifications. These sections were observed using a fluorescence microscope and images collected. From these images cell density and viability within the native cartilage matrix was determined by counting the live and dead cells in an identical defined area on each micrograph.

2.2.5 Histology and Immunohistochemistry

The formalin-fixed portion of each AC disc was embedded in paraffin using routine techniques. Serial sections 5µm thick were collected beginning at the apex of the defect in the injured explants. Sections were also taken from the centre of the uninjured explants to act as controls. Three sections from each dowel were subsequently stained using routine techniques with H&E and viewed using a light microscope. Images were collected to observe the morphology of the explants. Four sections from each dowel were stained using routine

immuonhistochemical techniques with an antibody specific to human collagen II [collagen II (2B1.5) (NeoMarkers)]. Antigen retrieval was accomplished with a 5 minute digestion with pepsin (1mg/ml) in 10 mM Tris-HCL pH 2.0 at 37ºC and the primary antibody was introduced in a 1:100 dilution. Staining was performed using the Vectastain ABC kit according to the manufacturer's specifications. Appropriate negative controls using the absence of the primary antibody and ligamentous tissue devoid of collagen II were included. All sections were reviewed for the presence of antibody staining using a light microscope and appropriate digital images were collected.

2.2.6 Statistical Analysis

The experiment was performed in triplicate and viability data were compared within each group and between groups using one way ANOVA and the software package SPSS.

2.3 Results

2.3.1 Cell Labeling, Adherence, and Migration

All of the cells were successfully labeled with the PKH 26 dye (Figures 2.2A and B) which is a membrane stain that fluoresces red. On day 0 explants in Group 3 (injured and seeded group) failed to demonstrate any immediate cellular adherence (Figure 2.2C). After 7 days it was observed that cells had securely adhered to the defects in all Group 3 samples even after washing with PBS. This was also seen in the 14 and 28 day explants (Figure 2.2D). Although dowels were

harvested from areas of the condyles with intact cartilage, these assessments were only made on gross visual examination. After sectioning it was clear that some dowels had some mild damage to the lamina splendens when viewed using the light microscope. It is interesting to note that when the lamina splendins was intact, there was no cellular adherence to the articular surface. In contrast, in those with lamina splendens damage, articular surface cell adhesion was apparent (Figure 2.2E and F).

At no point in the experiment was there evidence that any of the seeded cells had migrated into the acellular zone adjacent to the defect (Figure 2.3E and F). Nor was there any indication that the unlabelled cells in the native cartilage had migrated into this region.

2.3.2 Cell Density and Viability

Cell viability was determined at days 0, 14 and 28. When the Live/Dead assay is utilized it simultaneously detects both live and dead cells in the same tissue. Live cells take up the calcein-AM and metabolize it in the cytoplasm to produce a green fluorescent substrate which is retained by the intact membrane. Conversely, the dead cells allow the ethidium bromide to homodimer pass through the damaged cell membrane and stain the nucleic acid red (Figure 2.3A and B). There are two limitations to this method of detection. When a cell initially dies they are able to be stained but after a few days the cell debris will eventually be evacuated from the tissue. This is especially evident in the zone of cell loss adjacent to the defects (Figure 2.3C). Measurement of viable-cell

densities were obtained to determine if there was significant cell loss over the duration of the experiment (Figure 2.3D). Unfortunately, the dead cells fluoresced red which is the same color as the cultured cells labeled with PKH 26. This, however, was not a problem because adherence of the seeded cells was assessed first followed by staining for viability. The two processes were sequential and separate (Figure 2.3E and F).

There were viable cells adherent within the defect in Group 3 at 7, 14 and 28 days. Statistical comparison of the viable-cell density within the host cartilage matrix showed that there was no significant difference within Group 3 (injured and seeded) ($p=0.480$), Group 2 (injured) ($p=0.096$), and Group 1 (uninjured) ($p=0.404$) at each of the time points. This demonstrated that there was no significant cell loss within the groups *over time*. In contrast, a comparison of overall viable-cell density between the three groups showed there was a significant difference in overall viability *between* the three groups ($p<0.001$). Both of the injured groups had a significantly lower cell density when compared to the uninjured group. This was likely due to the apoptotic cell loss occurring in the injured explants and was not unexpected. There was no difference between the two injured Groups ($p = 0.157$).

2.3.3 Histology and Immunohistochemistry

Matrix formation by the implanted cells and the production of collagen type II was analyzed. In Group 3 (injured and seeded), a thin lattice-like network was produced surrounding the adherent cells as seen in both the H&E and

immunohistochemical stained images (Figure 2.4A and B). Immunohistochemical staining was most intense in the area immediately surrounding the seeded chondrocytes demonstrating collagen type II presence within the modest amount of *de novo* tissue. In Group 2 (injured), which did not receive any seeded chondrocytes, no new matrix was produced and the defects remained unchanged at all time points. There were no differences noted in the general architecture of the explant tissue when comparing the three groups aside from the obvious defects in the latter two groups.

2.4 Discussion

The development of a suitable AC construct at the site of injury is a difficult task in its own right but is complicated further by the requirement of effective integration of the construct into the adjacent host tissue. Furthermore, it has been recognized that the tissue adjacent to the defect is often decellularized due to a combination of necrosis and apoptosis[14]. It has recently been hypothesized that chondrocytes may be able to migrate into and repopulate this region of damaged matrix[20]. Indeed, it has been demonstrated that chondrocytes are capable of *in vitro* migration and deformation through pores as small as 8 μm when stimulated by the chemoattractant, IGF-1[24]. It has also been speculated that chondrocytes *in vivo* may be capable of migration through polarization of their catabolic processes in an attempt to generate a passage and migrate into regions of injury[20,24-26]. Archer et al.[14] attempted to elucidate this point by endeavoring to repopulate a decellularized matrix pellet with chondrocytes seeded onto the

surface. They were, however, unsuccessful but this was not unexpected due to a lack of chemostimulus in order to direct migration.

The results of this study have established a reproducible model which was used for the *in vitro* analysis of human AC defect repair. This work showed that seeded allogenous cells became adherent to injured areas, both in the experimental defects and also in regions where there was damage to the lamina splendens. Once adherent, the seeded chondrocytes produced a sparse matrix containing collagen II. However, it was noted that despite having viable cells within the host tissue presumably capable of a paracrine stimulus, seeded chondrocytes did not migrate into the region of cell death adjacent to the site of injury. This lack of cell migration was observed despite the use of osteoarthritic cartilage which represents a more permissive environment to cell movements due to the decreased matrix density and increased "pore size"[20]. Perhaps further enzymatic degradation of the existing matrix may be required to promote migration[27]. It is also possible that the paracrine stimulus provided by the cells within the explant was not sufficient to elicit a response in the seeded cells or that the 28 day time period was insufficient to allow significant cellular migration. Additionally, the culture environment of the explants needs to be further developed to duplicate the intraarticular conditions more closely.

This work also highlighted the importance of cartilage tissue manipulation and the effects this can have on cell viability within the tissue. Despite the deliberate minimal use of low pressure and friction to create the AC defects a zone of cell loss adjacent to the defect still developed. This has been seen in other

work[14,19,28]and it is clear that a greater understanding of the microenvironment at the host-tissue interface is essential if effective construct integration is to be achieved. Additionally, this work also serves as a reminder to surgeons that AC cannot be treated with impunity but in contrast must be treated with the utmost respect. Any invasion of the AC is likely to cause more harm than good and the common use of arthroscopic shavers to "smooth" AC should be reviewed.

This study was seen as the first of several designed to identify the major problems associated with using implanted cells to repair AC as well as to direct improvements in the methodology. Future improvements might include development of a model which more closely mimics the intraarticular environment with a particular emphasis on appropriate oxygen tension and applied biomechanical forces. Also, now that adherence of seeded chondrocytes has been demonstrated using this model, future work will focus on the use of matrices and bimolecules overlying these adherent cells to see if cellular migration and *de novo* matrix formation can be encouraged from the base of the defect towards the surface. This model also shows promise for use in further work with enzymatic preparations[29] of cartilage prior to construct insertion and also for anti-oxidant treatments[14] which could limit the apoptotic cell loss adjacent to the AC injury.

With these limitations and future directions in mind, this model shows promise as a reproducible *in vitro* tool for the investigation of articular cartilage defect repair. Although there was no apparent cell migration observed, this model could be used to study the interaction of implanted cells and host tissue and may

provide insight to better optimize defect preparation to promote cell adherence and graft integration in AC repair.

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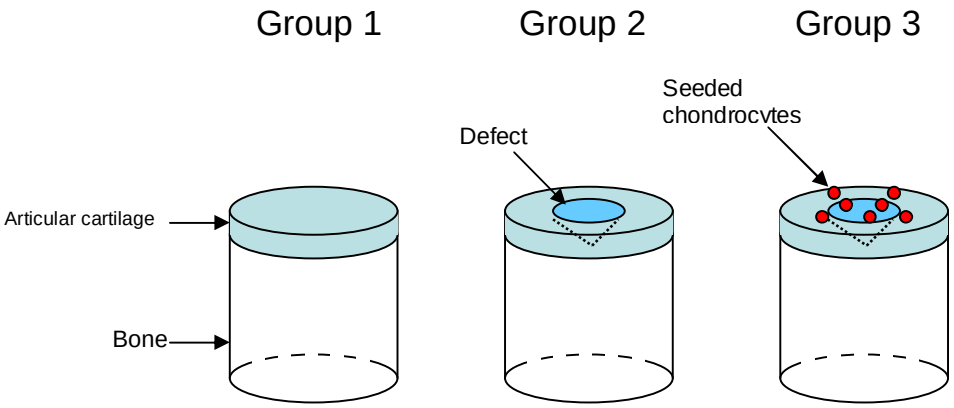


Figure 2.1. Schematic outlining the experimental groups. Group 1 which is the uninjured dowel for control purposes, Group 2 the injured dowel which serves as a negative control, and Group 3 the injured and seeded group with chondrocytes implanted at the site of injury.

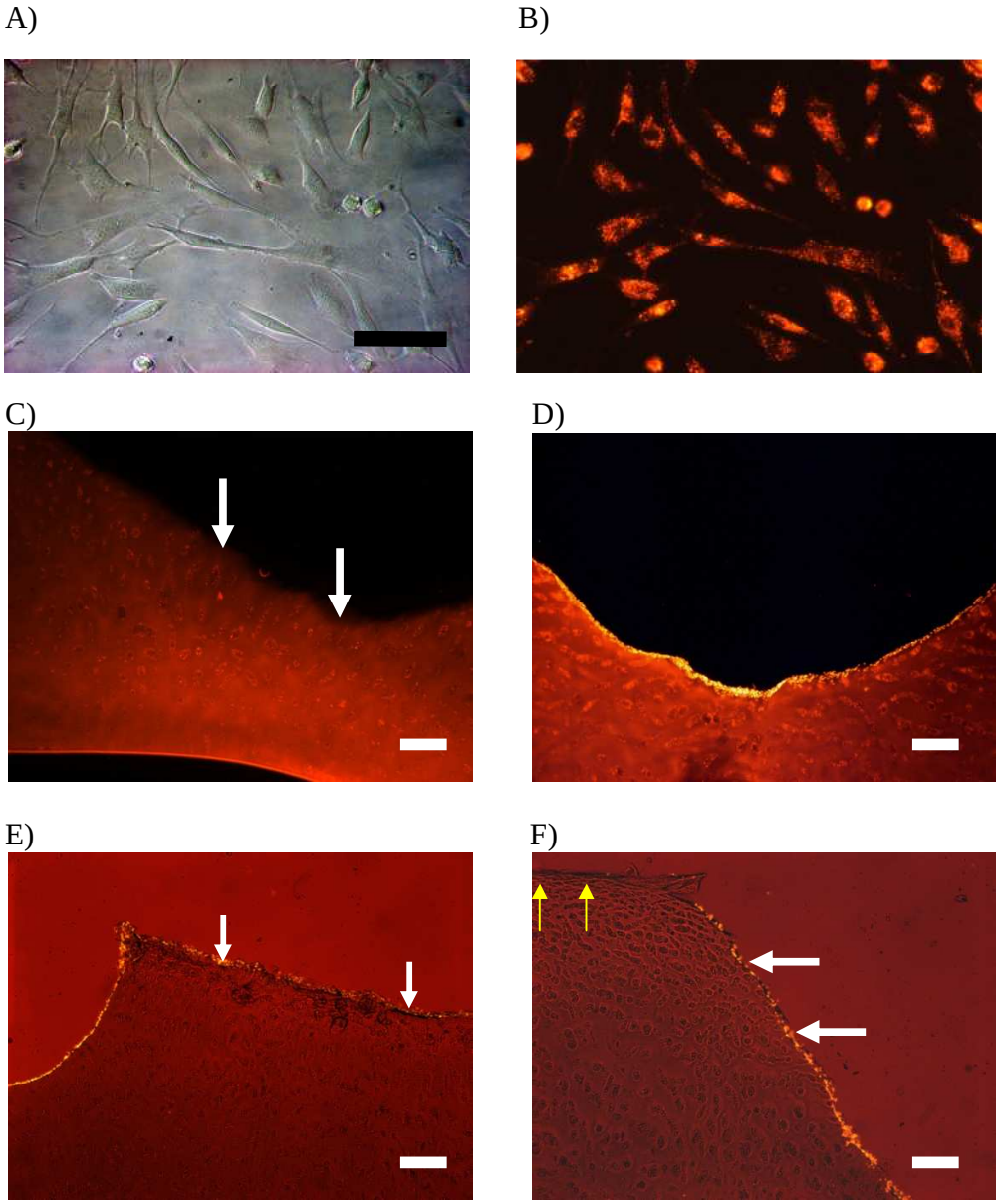


Figure 2.2. A) Light micrograph of cultured chondrocytes prior to implantation (Bar = 50µm). B) Same image as A) but viewed using fluorescence showing successful staining of cells with PKH 26. C) Fluorescence image of day 0 defect demonstrating no labeled cell adherence (white arrows indicate defect surface). D) Fluorescence image of 7 day defect demonstrating cell adherence within the defect. E) Fluorescence image of 7day dowel showing cells adhering to the AC surface at a region of disruption of the lamina splendins (indicated by the white arrows) Note that there is also adherence within the defect. F) Fluorescence image of a day 7 dowel showing adherence of the cultured chondrocytes to the defect (white arrows) and not to an adjacent area of in tact lamina splendins (yellow arrows). (Bar = 100µm)

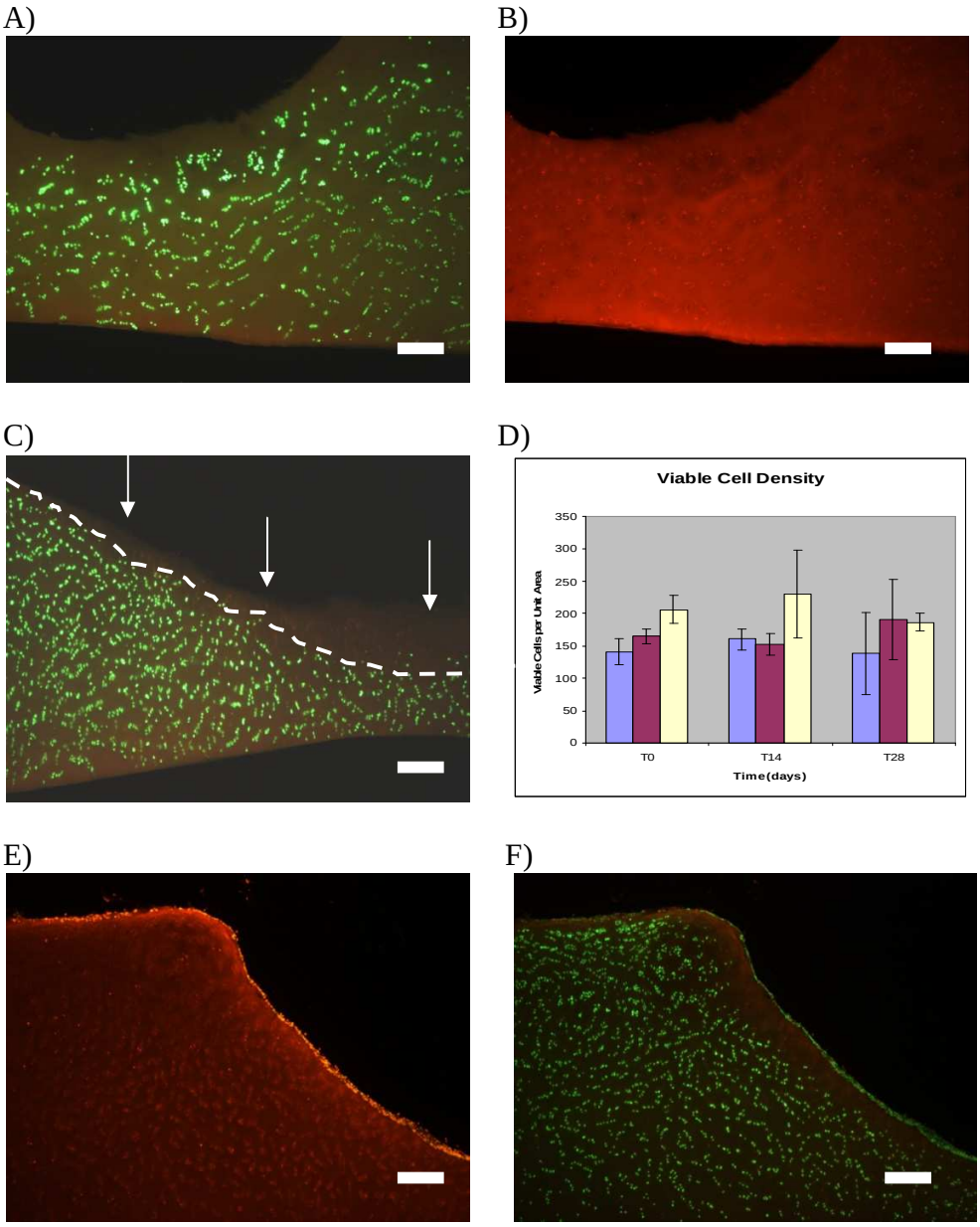


Figure 2.3. A) Fluorescence image showing live cells which fluoresce green. B) Same section as A) but viewed with fluorescence for the dead cells. Note that there were few dead cells present.. C) Fluorescence image showing acellular band adjacent to the defect site. The arrows indicate the defect surface and the dashed line defines the viable cell boundary. D) A graph comparing the cell densities in each of the three experimental groups over time. Note that there is no significant difference within each of the groups but there was a difference between the dowels with an injury and those in the uninjured group (blue = Injured and Seeded, purple = Injured, white = uninjured) E) A fluorescence image showing a seeded defect demonstrating chondrocyte adhesion prior to viability staining. F) Fluorescence image showing the same section viewed in E) but stained with viability stained and viewed with the appropriate filter (Bar = 100µm)

A) B)

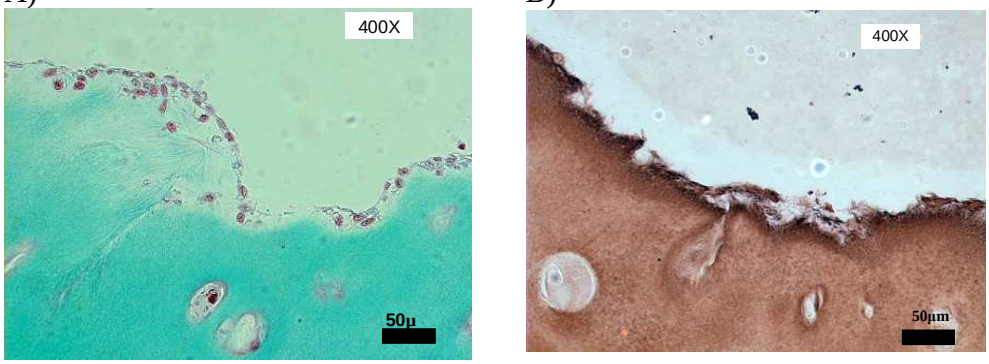


Figure 2.4. A) A light micrograph of the injury site of a 28 day dowel stained with H&E demonstrating a thin layer of matrix formation surrounding seeded chondrocytes. B) A light micrograph of the injury site of a 28 day dowel stained with DAB demonstrating collagen II deposition around the implanted chondrocytes. The more intense brown staining represents increased collagen II production. (Bar = 50µm)

2.5 Reference List

1. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage*. **10**, 432-463 (2002).

2. Buckwalter,J.A. Sports, joint injury, and posttraumatic osteoarthritis. *J. Orthop. Sports Phys. Ther.* **33**, 578-588 (2003).

3. Buckwalter,J.A., Saltzman,C. & Brown,T. The impact of osteoarthritis: implications for research. *Clin. Orthop. Relat Res.* S6-15 (2004).

4. Buckwalter,J.A. Articular cartilage injuries. *Clin. Orthop. Relat Res.* 21-37 (2002).

5. Alford,J.W. & Cole,B.J. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am. J. Sports Med.* **33**, 295-306 (2005).

6. Reginster,J.Y. The prevalence and burden of arthritis. *Rheumatology. (Oxford)* **41 Supp 1**, 3-6 (2002).

7. Elders,M.J. The increasing impact of arthritis on public health. *J. Rheumatol. Suppl* **60**, 6-8 (2000).

8. Brooks,P.M. Impact of osteoarthritis on individuals and society: how much disability? Social consequences and health economic implications. *Curr. Opin. Rheumatol.* **14**, 573-577 (2002).

9. Meyer,U., Buchter,A., Wiesmann,H.P., Joos,U. & Jones,D.B. Current strategies for articular cartilage repair. *Eur. Cell Mater.* **9**, 39-49 (2005).

10. Hunziker,E.B., Quinn,T.M. & Hauselmann,H.J. Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis. Cartilage*. **10**, 564-572 (2002).

11. Buckwalter,J., Simon,S. & Einhorn,T. Orthopedic Basic Science. Biology and Biomechanics of the Musculoskeletal System. 2nd Edition. (2000).

12. Hunziker,E.B., Kapfinger,E. & Geiss,J. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. *Osteoarthritis. Cartilage*. **15**, 403-413 (2007).

13. Quinn,T.M., Hunziker,E.B. & Hauselmann,H.J. Variation of cell and matrix morphologies in articular cartilage among locations in the adult human knee. *Osteoarthritis. Cartilage*. **13**, 672-678 (2005).

14. Archer,C.W., Redman,S., Khan,I., Bishop,J. & Richardson,K. Enhancing tissue integration in cartilage repair procedures. *J Anat.* **209**, 481-493 (2006).

15. Langer,R. & Tirrell,D.A. Designing materials for biology and medicine. *Nature* **428**, 487-492 (2004).

16. Griffith,L.G. & Naughton,G. Tissue engineering--current challenges and expanding opportunities. *Science* **295**, 1009-1014 (2002).

17. Lutolf,M.P. & Hubbell,J.A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotechnol.* **23**, 47-55 (2005).

18. Quinn,T.M. & Hunziker,E.B. Controlled enzymatic matrix degradation for integrative cartilage repair: effects on viable cell density and proteoglycan deposition. *Tissue Eng* **8**, 799-806 (2002).

19. Redman,S.N., Dowthwaite,G.P., Thomson,B.M. & Archer,C.W. The cellular responses of articular cartilage to sharp and blunt trauma. *Osteoarthritis. Cartilage*. **12**, 106-116 (2004).

20. Morales,T.I. Chondrocyte moves: clever strategies? *Osteoarthritis. Cartilage*. **15**, 861-871 (2007).

21. van der Kraan,P.M., Buma,P., van Kuppevelt,T. & van den Berg,W.B. Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. *Osteoarthritis. Cartilage.* **10**, 631-637 (2002).

22. Qiu,W. *et al.* Outgrowth of chondrocytes from human articular cartilage explants and expression of alpha-smooth muscle actin. *Wound. Repair Regen*. **8**, 383-391 (2000).

23. OUTERBRIDGE,R.E. The etiology of chondromalacia patellae. *J. Bone Joint Surg. Br.* **43-B**, 752-757 (1961).

24. Chang,C., Lauffenburger,D.A. & Morales,T.I. Motile chondrocytes from newborn calf: migration properties and synthesis of collagen II. *Osteoarthritis. Cartilage*. **11**, 603-612 (2003).

25. Lee,G.M. & Loeser,R.F. Cell surface receptors transmit sufficient force to bend collagen fibrils. *Exp. Cell Res.* **248**, 294-305 (1999).

26. Maniwa,S. *et al.* Effects of hyaluronic acid and basic fibroblast growth factor on motility of chondrocytes and synovial cells in culture. *Acta Orthop. Scand.* **72**, 299-303 (2001).

27. Qiu,W. *et al.* Outgrowth of chondrocytes from human articular cartilage explants and expression of alpha-smooth muscle actin. *Wound. Repair Regen.* **8**, 383-391 (2000).

28. Tew,S.R., Kwan,A.P., Hann,A., Thomson,B.M. & Archer,C.W. The reactions of articular cartilage to experimental wounding: role of apoptosis. *Arthritis Rheum.* **43**, 215-225 (2000).

29. Hunziker,E.B. & Rosenberg,L.C. Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane. *J. Bone Joint Surg. Am.* **78**, 721-733 (1996).

CHAPTER THREE

ALTERATIONS IN GENE EXPRESSION IMMEDIATELY AFTER REMOVAL OF ARTICULAR CHONDROCYTES FROM THEIR NATIVE MATRIX

Alterations in gene expression immediately after removal of articular chondrocytes from their native matrix

3.1 Introduction

Articular cartilage (AC) is an avascular tissue which has a poor inherent healing capacity following injury. The attempts of the natural repair process result in the formation of biomechanically inferior fibrocartilage which eventually breaks down leading to progressive joint degeneration and osteoarthritis (OA)[1-6]. The social and economic impacts of OA on society are enormous, highlighted by the fact that OA is second only to ischemic heart disease as a cause of work-related disability in males over 50 years of age[7-11]. Therefore, repair of AC focal defects prior to joint degeneration is of paramount concern.

The challenge involved with development of AC repair techniques is substantial with several different techniques currently being employed with variable results[2,6]. Arguably the current gold-standard for focal AC defect repair is autologous chondrocyte implantation (ACI)[12]. This process involves the removal of a small portion of normal AC from a non-weight bearing region of the joint during an initial surgical procedure, harvest and expansion of the chondrocyte number *in vitro*, followed by implantation of the expanded chondrocyte population into the AC defect under cover of a periosteal patch[13-15]. Despite criticisms of this technique,[2,16,17]cartilage repair procedures have been performed in this manner for over a decade with studies suggesting real patient benefit[12]. However, it is recognized that improvements are required to make this technique more reliable, durable, and applicable to larger defects[18,19].

One problem associated with ACI is that it utilizes chondrocytes expanded in monolayer culture resulting in loss of the differentiated chondrocyte phenotype[20-22]. This process, termed de-differentiation, is characterized primarily by the change in expression of AC matrix-associated collagen type II to the fibrocartilage-associated collagen type I[19]. To overcome this problem, several culture systems have been developed which attempt to partially prevent or reverse this change in phenotype through retention or restoration of the rounded chondrocyte shape[23], medium supplementation with biomolecules[24], alteration in oxygen tension[25,26], or growth in high-density cultures[27]. Unfortunately, these systems aimed at chondrocyte re-differentiation appear unable to return the chondrocytes fully to their *in vivo* functional state and result instead in the generation of chondrocytes that produce an inferior fibrocartilagenous or "hyaline-like" tissue that is not durable[28-30].

Observation and measurement of gene expression during chondrocyte culture is not new and has been used extensively to identify the basic genotype for implanted cells. Usually transcription levels of passage 0 cells have been used as the baseline for comparison with subsequent transcription values. However, it is entirely possible that the chondrocyte phenotype might be significantly altered prior to harvest at passage 0, especially during initial digestion, which would nullify chondrocyte phenotype at passage 0 as being representative of normal values. An accurate representation of baseline *in situ* gene expression is essential if appropriate values are to be used for comparison and development of chondrocytes with appropriate characteristics suitable for implantation.

Accordingly, this project was designed to determine the pattern of chondrocyte gene expression from *in situ* through to passage 7 focusing on the levels of collagen type I, collagen type II and aggrecan gene expression. It was hypothesized that this approach would provide better baseline data especially if changes in the expression of these genes occurred prior to harvesting the cells at passage 0.

3.2 Methods

Normal human AC was collected from the distal femur of three human cadavers (Human Organ Procurement and Exchange; HOPE program) with permission from the University of Alberta Hospital ethics committee. Chondrocytes were harvested from each patient, plated in monolayer and expanded through to passage 7. Messenger RNA was extracted from the cells both *in situ* and at the end of each passage and analyzed using real time PCR (RT-PCR). Collagen type I protein expression was detected with immunofluorescence in the passage 0 cell population. The experiment was performed in triplicate and the methods are detailed below.

3.2.1 Chondrocyte isolation and culture

Chondrocytes were obtained from AC on the weight-bearing portion of the femoral condyles in fresh human cadavers. Strips of full-thickness cartilage (~1cm wide) were removed from the underlying subchondral bone using a sterile scalpel. The strips were digested for 6 hours at 37ºC in collagenase type 1A

(Sigma C-9891) (1mg/ml) in serum free DMEM-F12 and 1 ml of the solution was utilized for each gram of cartilage. The chondrocytes were collected through centrifugation at 400xg for 5 minutes and the cell pellet was washed twice with serum-containing medium. Chondrocytes were then plated in monolayer and passaged (1:2) when approaching confluence. At each passage chondrocytes were released from the plates with 0.25% Trypsin + EDTA (Lonza). Half of the cells were pelleted and stored at -80ºC to await analysis while the remainder were carried through to the next passage. The cells were grown at 37ºC and 5% CO_2 in Chondrocyte Growth Medium (CGM) (Lonza) which was changed every 3-4 days. This process was repeated until passage 7 was completed.

3.2.2 RNA isolation and real time polymerase chain reaction (RT-PCR)

RNA was extracted from the chondrocyte cell lines utilizing the SV Total RNA Isolation System (Promega) according to the manufacturer's specifications. RNA isolation from the cartilage was successfully achieved after grinding the tissue into a powder in liquid nitrogen using the Spex® Sample Prep freezer mill (model 6770). The ground cartilage was processed using the Total RNA Fatty and Fibrous Tissue Pack (Bio-Rad) according to the manufacturer's specifications with the following modifications: in the initial step 50mg of tissue was added to 1 ml of PureZol® because this was found to improve yields in previous experiments in our lab. The purity and quantity of RNA was determined in the usual manner through utilization of the $OD_{260/280}$ ratio.

cDNA was generated from all samples with the Superscript III 1st-strand synthesis system (Invitrogen) according to the manufacturers specifications. The reagents for the RT-PCR were taken from the QuantiTect SYBR Green PCR kit (Qiagen). The primers utilized are recorded in Table 3.1. Quantification and analysis for each of the reactions was carried out using the MYiQ single-color real-time PCR detection system (BioRad). The PCR conditions were as follows: 94°C for 15 seconds, annealing (GAPDH and Aggrecan 60°C; Collagen I and II 58°C) for 20 seconds, 72°C for 10 seconds for 45 cycles. The comparative C_T method was employed to provide relative quantification of gene expression.

3.2.3 Collagen Type I immunofluorescence

Immediately following collagenase digestion of the AC, 5 x 10^4 chondrocytes were seeded into LAB-TEK II chamber slides (Nalge-Nunc) and incubated for 72 hrs in CGM to allow the cells to attach and produce extracellular matrix molecules. Cells were washed in PBS and fixed with 100% methanol. The cells were blocked with milk followed by primary antibody (Collagen type I AB758, Chemicon International) exposure at a 1:100 dilution. The cells were exposed to a series of washes (PBS, PBST and PBS) and incubated in a 1:100 dilution of secondary antibody (anti-mouse Alexa 488-labeled antibody). Cells were exposed to another series of 5 min washes (PBS, PBST and PBS) and viewed using a Leica fluorescence microscope. A standard fibroblast cell-line which produces collagen type I was used as a positive control and preparations without the application of a primary antibody were used as a negative control.

3.3 Results

3.3.1 Collagen Type I

The expression of collagen type I transcripts from cells obtained directly *in situ* was much lower than that found at passage 0 in all patients and occurred between chondrocyte digestion and harvest of the cells. This up-regulation of gene expression was substantial ($\sim 2^{10}$) when compared with levels obtained *in situ* and was maintained through all passages. Although there was variability between patients with respect to the degree of gene expression up-regulation, the overall pattern was similar in all cases (Figure 3.1).

Observation of the images showing sites of immunofluorescence for collagen type I (Figure 3.2) confirmed the presence of the protein in passage 0 cells. However, it is interesting to note that not all of the cells showed evidence of collagen type I production at this time. Furthermore, differing intensities of fluorescence within the cells suggested that the cells were producing the protein in varying amounts. This was in sharp contrast to the image of the fibroblast control cells which demonstrated a uniform fluorescence in all cells as was expected (Figure 3.2).

3.3.2 Collagen Type II

The relative gene expression of collagen type II was up-regulated following matrix digestion and harvest at passage 0 when compared to the levels obtained *in situ*. This up-regulation was, however, on a much smaller ($\sim 2^{1}$) scale than that for collagen type I already described and, furthermore did not persist. In

fact, by the end of passage 2 the gene expression levels were already below those *in situ* and continued to decrease until by passage 7 the level of collagen type II transcript was almost undetectable(~2^{-20}) (Figure 3.3). Again the level of changes in gene expression varied from subject to subject but the same pattern of gene expression was present in all samples, increasing initially and then rapidly decreasing.

3.3.3 Aggrecan

In sharp contrast, the pattern of relative mRNA expression of aggrecan was much less variable. Overall, there was a small decrease (~2^{-3}) in expression from the levels obtained *in situ* through all passages This pattern was the same in all patients and one sample is presented in Figure 3.4 as an example.

3.4 Discussion

The results of this study have confirmed those of several earlier studies which have shown the rapid loss of genotype accompanying chondrocyte culture[20-22]. However, this current study has also identified the level of gene expression for the chondrocytes while *in situ* and found these values to be different to those found in the early stages of culture. Consequently, these values are more realistic for use as a baseline for comparison during subsequent passaging in culture and in the development of chondrocytes more appropriate for implantation. Earlier studies have used the values obtained at passage 0 as the baseline for comparison and these are clearly different to the initial values seen *in*

situ. This appears to be the first time that the gene expression levels based on *in situ* values have been reported in the English literature and provides new, significant insights into the loss of genotype of chondrocytes during culture especially when compared to subsequent values obtained during passaging. The new information is especially important for the development of cultured chondrocytes needed for repair of AC where the new cells are required to express appropriate chondrocyte characteristics prior to implantation. Presumably these characteristics are essential for the implanted cells if the creation of new AC matrix and successful integration into adjacent existing tissue is to be achieved. Gene expression levels determined at the end of passage 0 are clearly inappropriate for use as baseline data for this purpose as they are very different to those obtained *in situ*.

The results from this study have shown that when chondrocytes are released from their surrounding matrix there is an immediate and rapid change in gene expression, so much so that they have lost significant chondrocyte characteristics at least by passage 0 if not sooner. By the end of passage 0, gene expression levels for collagen type I have risen ~1000x while levels for collagen type II have also risen but only by ~10x when compared to the levels obtained from chondrocytes *in situ*. In contrast, gene expression levels for aggrecan have decreased by ~10x in the same time period. These changes in gene expression levels, especially the large changes associated with collagen type I, suggest strongly that the cultured cells can no longer be considered as chondrocytes and should be seen only as chondrocyte-like at best. If cultured cells are to be

implanted at sites of injury it would seem appropriate to suggest that the gene expression values should be returned to those found *in situ* prior to implantation if any tissue repair is to be eventually successful. However, completely successful methods for returning gene expression levels to those found *in situ* remain elusive and are the focus of intensive research efforts[23-27]. There are many potential reasons for these changes in gene expression levels and it is probable that a major contributor might be the reduced contact with adjacent tissues while being cultured when compared to that found in normal matrix. It is expected that a better understanding of the underlying mechanism would lead directly to better methods for maintenance or recovery of the necessary chondrocyte characteristics.

The pattern of change in gene expression levels demonstrated in this study by the cultured chondrocytes during passaging is similar to that described elsewhere by others[31-34]. This has led to attention being focused on the apparent gradual loss of chondrocyte phenotype over a period of two weeks[20,23,35,36] following matrix digestion. However, results from this study have shown that the loss of the chondrocyte phenotype occurred prior to harvest at passage 0 especially with the enormous (~1000x) up-regulation of collagen type I gene expression immediately after digestion of the cartilage matrix. The inclusion of values of gene expression obtained for cells *in situ* as the initial starting values rather than those obtained at passage 0 significantly changes any description of the pattern of gene expression during culture. This is especially so with the initial up-regulation of collagen types I and II expression which is not evident if the *in*

situ values are excluded. The up-regulation of mRNA levels of collagen type II in chondrocytes immediately following matrix digestion is not entirely unexpected because it is reasonable to suggest that an attempt at repair involving mechanisms of collagen type II production would be made by the cells once they have been removed from their matrix. Similarly, it is not unreasonable to suggest that the subsequent inability to create a recognizable matrix in the unfamiliar culture conditions would continue to affect gene expression of collagen type II. This initial up-regulation is an important finding and must be included in any description of development in cultured cells and attempts to re-establish chondrocyte genotype.

Traditionally, a combination of the production of collagen type II and an absence of collagen type I has loosely defined an articular chondrocyte once it is removed from its native matrix[19]. It is thought that the production of collagen type I is detrimental to the formation of durable AC[28-30] which contains predominantly collagen type II in normal conditions[37]. While the tensile strength and fibril diameter of collagen type I is comparable to collagen type II[38,39], it is thought that the poor integrity of fibrocartilage repair tissue (consisting of mainly collagen type I) is likely due to the inability of collagen I to interact and retain aggrecan in the same manner as the native collagen type II within the matrix[38]. The presence of collagen type I would lead to a decreased hydrostatic effect and a loss of the tissue's compressive modulus.

It is interesting to compare the patterns of gene expression of collagen types I and II during culture and consider what this could represent in relation to

specific cells. In this respect, the level of production of collagen type I in cultured cells appears to increase upon release of the cells from their surrounding matrix and then remains constant during subsequent passaging. In contrast, the levels for collagen type II decline steadily after their initial up-regulation without a parallel increase in collagen type I production. By necessity, the results of this study describe average values for the cell population and not values for individual cells and while population values might change, values for individual cells might be different. Several possible explanations exist to explain the changes in population gene expression levels. These results suggest that while some cells commence production of collagen type I following release from the matrix, other cells reduce collagen type II production but do not commence production of collagen type I. This suggestion is supported by the limited number of cells seen to be exhibiting collagen type I (fluorescence staining) and the large number of cells with no fluorescence. It is not clear whether a single cell can produce both collagen types I and II simultaneously but these results suggest not and provides information important for consideration for re-differentiation. It is entirely possible that two separate populations of cells are established following release from the AC matrix with some cells producing collagen type I while others simply stop producing collagen type II. Perhaps these cells should be isolated from each other and only those producing collagen type II should be used if successful implantation is to be achieved. Of course, it might be more advantageous overall if methods were developed to preserve chondrocyte phenotype very early in culture, even during initial matrix digestion, so that collagen type I up-regulation is prevented

especially if the up-regulation is irreversible. This problem provides much thought for future work.

The results of this study have also emphasized that the current definition of a chondrocyte as "being the only cell within cartilage"[40] needs to be revisited. While useful in the past, this limited definition cannot be applied to cells once they have been removed from their matrix because their character changes so quickly and dramatically. Other groups have attempted to define cells in culture obtained from AC based loosely on the expression of a limited number of matrix components, mainly collagen type II, aggrecan and a lack of collagen type I gene expression[18,19], but any such definition remains inadequate for research studies designed to develop cells for implantation at sites of AC injury. A much broader, expanded definition which includes a combination of genomic, proteomic and metabolic parameters that can standardize cell description between research groups needs to be established if significant progress is to be made.

Over a decade ago it was written "that cartilage does not yield its secrets easily and that inducing cartilage to heal is not simple"[41]. Unfortunately, this lament continues to hold true today. There remains a significant amount about chondrocyte biology that remains unknown and it is becoming increasingly clear that for greater progress to be made understanding and communication within the field must be improved so that apples are not being compared to oranges.

Gene	Size (bp)	Strand	Sequence(5´-3´)
Type II Collagen	257	**F**	GACAATCTGGCTCCCAAC
		R	ACAGTCTTGCCCCACTTAC
Type I Collagen	105	**F**	AGGTGCTGATGGCTCTCCT
		R	GGACCACTTTCACCCTTGT
Aggrecan	85	**F**	TCGAGGACAGCGAGGCC
		R	TCGAGGGTGTAGCGTGTAGAGA
GAPDH	189	**F**	TGGTATCGTGGAAGGACTCATGAC
		R	ATGCCAGTGAGCTTCCCGTTCAGC

Table 3.1. Primers for RT-PCR.

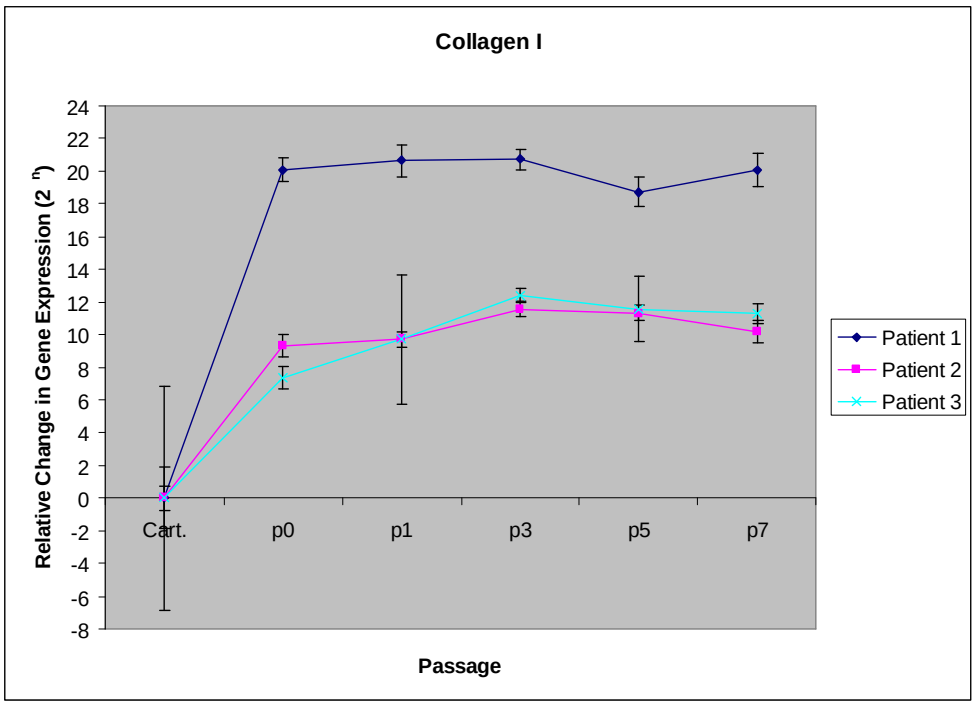


Figure 3.1. The relative change in gene expression of collagen type I in each of the three patient subjects. Note that in each case there is an immediate up-regulation of gene expression which is maintained through all passages.

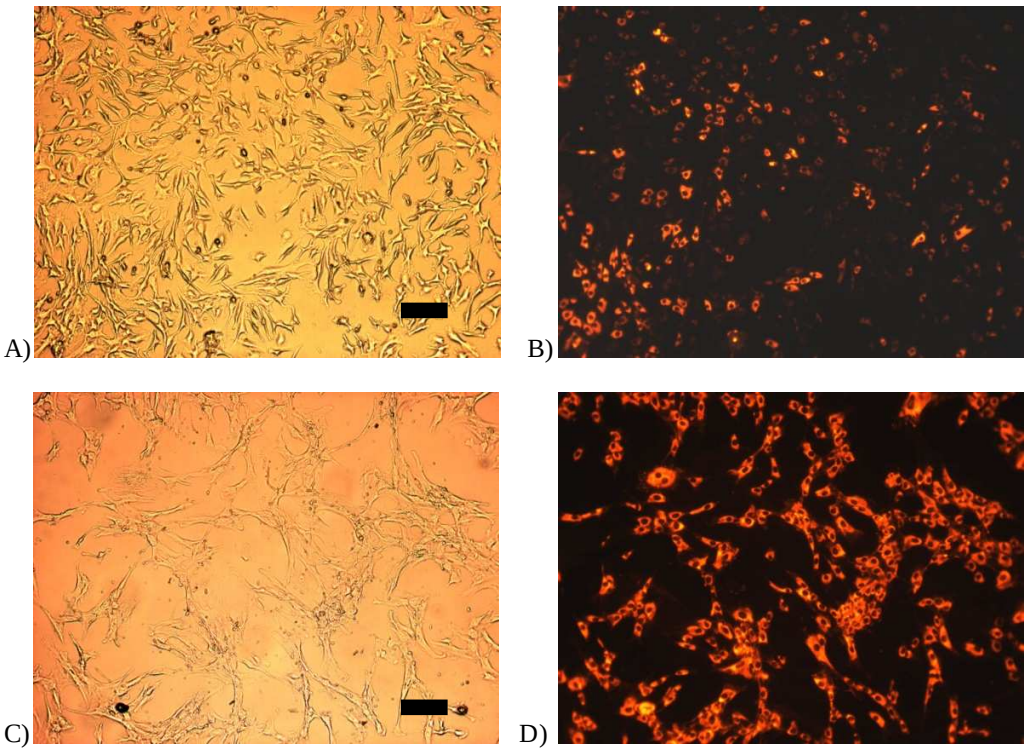


Figure 3.2. Microscope images illustrating collagen I immunofluorescence. A) Light microscope image of passage 0 chondrocytes in monolayer culture. B) Fluorescence micrograph of the same cells depicted in image A. Note that only a portion of the cells have expressed collagen type I and that its distribution is variable as demonstrated by differences in the degree of fluorescence. C & D) These images represent similar micrographs but of fibroblast cells acting as controls. The fluorescence is present in every cell and is much more uniformly distributed (Bar = 50µm).

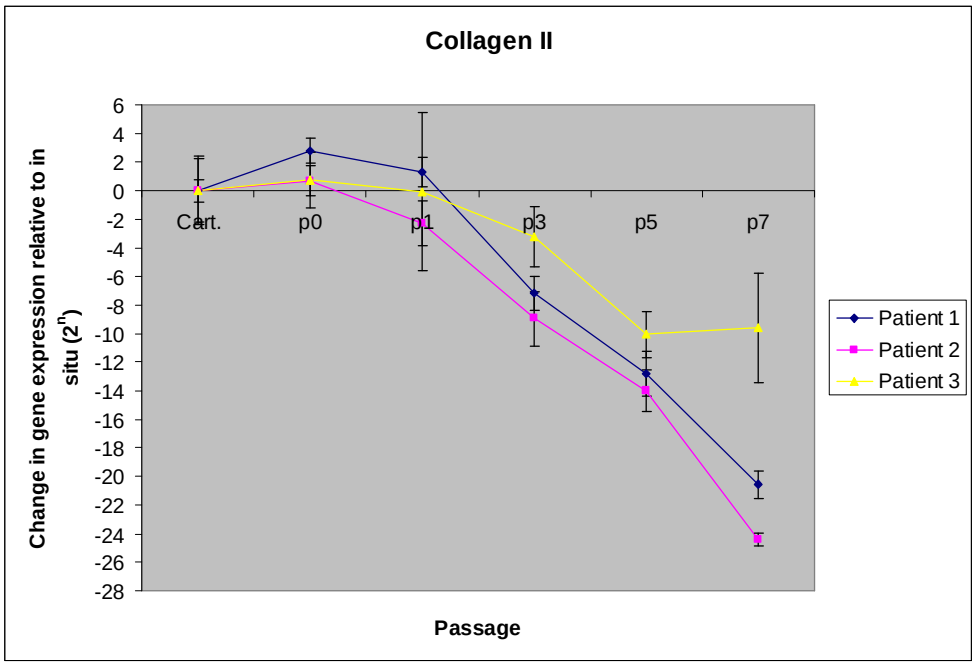


Figure 3.3. The relative change in gene expression of collagen type II in each of the three patients. Notice that overall there was a transient increase in collagen type II expression followed by a down-regulation of expression which continued with subsequent passages. In each case the mRNA expression of collagen type II has decreased significantly to be below that of the *in situ* expression by passage 2.

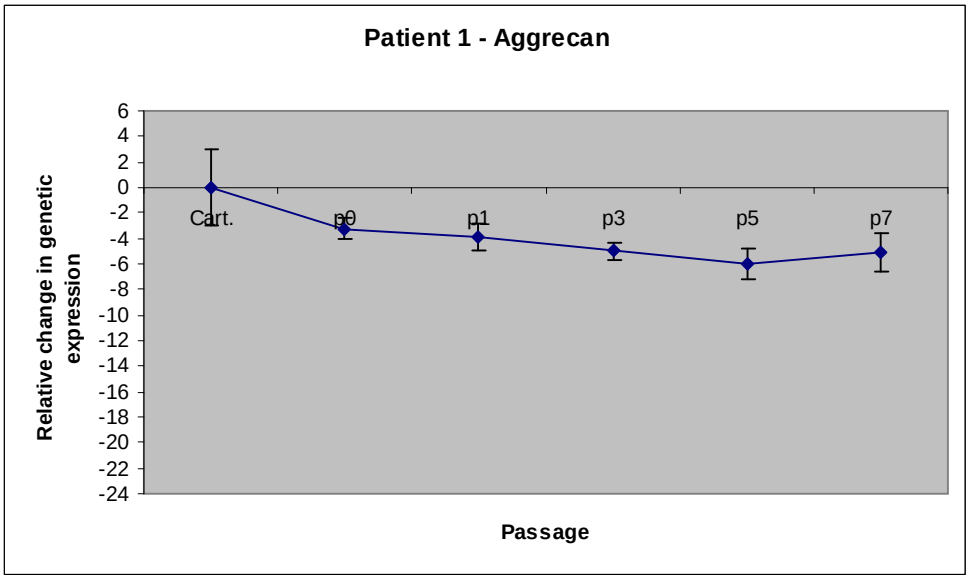


Figure 3.4. A typical expression profile for aggrecan during chondrocyte expansion. Note that there is a gradual decline with each passage.

3.5 Reference List

1. Buckwalter,J., Simon,S. & Einhorn,T. Orthopedic Basic Science. Biology and Biomechanics of the Musculoskeletal System. 2nd Edition. (2000).

2. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage*. **10**, 432-463 (2002).

3. Buckwalter,J.A. & Martin,J.A. Sports and osteoarthritis. *Curr. Opin. Rheumatol.* **16**, 634-639 (2004).

4. Ritchie,P.K. & McCarty,E.C. Surgical management of cartilage defects in athletes. *Clin. Sports Med.* **24**, 163-174 (2005).

5. Meyer,U., Buchter,A., Wiesmann,H.P., Joos,U. & Jones,D.B. Current strategies for articular cartilage repair. *Eur. Cell Mater*. **9**, 39-49 (2005).

6. Alford,J.W. & Cole,B.J. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am. J. Sports Med.* **33**, 295-306 (2005).

7. Reginster,J.Y. The prevalence and burden of arthritis. *Rheumatology. (Oxford)* **41 Supp 1**, 3-6 (2002).

8. Elders,M.J. The increasing impact of arthritis on public health. *J. Rheumatol. Suppl* **60**, 6-8 (2000).

9. Buckwalter,J.A., Saltzman,C. & Brown,T. The impact of osteoarthritis: implications for research. *Clin. Orthop. Relat Res*. S6-15 (2004).

10. Brooks,P.M. Impact of osteoarthritis on individuals and society: how much disability? Social consequences and health economic implications. *Curr. Opin. Rheumatol.* **14**, 573-577 (2002).

11. Lawrence,R.C. *et al.* Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. *Arthritis Rheum.* **41**, 778-799 (1998).

12. Peterson,L., Brittberg,M., Kiviranta,I., Akerlund,E.L. & Lindahl,A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. *Am. J. Sports Med.* **30**, 2-12 (2002).

13. Brittberg,M., Peterson,L., Sjogren-Jansson,E., Tallheden,T. & Lindahl,A. Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments. *J Bone Joint Surg. Am.* **85-A Suppl 3**, 109-115 (2003).

14. Brittberg,M., Tallheden,T., Sjogren-Jansson,B., Lindahl,A. & Peterson,L. Autologous chondrocytes used for articular cartilage repair: an update. *Clin. Orthop. Relat Res.* S337-S348 (2001).

15. Lindahl,A., Brittberg,M. & Peterson,L. Cartilage repair with chondrocytes: clinical and cellular aspects. *Novartis. Found. Symp.* **249**, 175-186 (2003).

16. Driesang,I.M. & Hunziker,E.B. Delamination rates of tissue flaps used in articular cartilage repair. *J. Orthop. Res.* **18**, 909-911 (2000).

17. Grande,D.A., Pitman,M.I., Peterson,L., Menche,D. & Klein,M. The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. *J. Orthop. Res.* **7**, 208-218 (1989).

18. Yang,K.G. *et al.* Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. *Tissue Eng* **12**, 2435-2447 (2006).

19. Hardingham,T.E., Oldershaw,R.A. & Tew,S.R. Cartilage, SOX9 and Notch signals in chondrogenesis. *J Anat.* **209**, 469-480 (2006).

20. Benya,P.D., Padilla,S.R. & Nimni,M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313-1321 (1978).

21. Benya,P.D., Padilla,S.R. & Nimni,M.E. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. *Biochemistry* **16**, 865-872 (1977).

22. Cheung,H.S., Harvey,W., Benya,P.D. & Nimni,M.E. New collagen markers of 'derepression' synthesized by rabbit articular chondrocytes in culture. *Biochem. Biophys. Res. Commun.* **68**, 1371-1378 (1976).

23. Benya,P.D. & Shaffer,J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**, 215-224 (1982).

24. Francioli,S.E. *et al.* Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: Relevance for Automated Bioreactor Systems. *Tissue Eng* (2007).

25. Domm,C., Schunke,M., Steinhagen,J., Freitag,S. & Kurz,B. Influence of various alginate brands on the redifferentiation of dedifferentiated bovine articular chondrocytes in alginate bead culture under high and low oxygen tension. *Tissue Eng* **10**, 1796-1805 (2004).

26. Domm,C., Schunke,M., Christesen,K. & Kurz,B. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. *Osteoarthritis. Cartilage.* **10**, 13-22 (2002).

27. Schulze-Tanzil,G. *et al.* Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. *Cell Tissue Res.* **308**, 371-379 (2002).

28. Yang,I.H. *et al.* Comparison of phenotypic characterization between "alginate bead" and "pellet" culture systems as chondrogenic differentiation models for human mesenchymal stem cells. *Yonsei Med. J.* **45**, 891-900 (2004).

29. Knutsen,G. *et al.* A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. *J. Bone Joint Surg. Am.* **89**, 2105-2112 (2007).

30. Barbero,A. *et al.* Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. *Osteoarthritis. Cartilage.* **12**, 476-484 (2004).

31. Tallheden,T. *et al.* Gene expression during redifferentiation of human articular chondrocytes. *Osteoarthritis. Cartilage.* **12**, 525-535 (2004).

32. Barlic,A., Drobnic,M., Malicev,E. & Kregar-Velikonja,N. Quantitative analysis of gene expression in human articular chondrocytes assigned for autologous implantation. *J Orthop. Res.* (2008).

33. Darling,E.M. & Athanasiou,K.A. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop. Res.* **23**, 425-432 (2005).

34. Takahashi,T. *et al.* Three-dimensional microenvironments retain chondrocyte phenotypes during proliferation culture. *Tissue Eng* **13**, 1583-1592 (2007).

35. Marlovits,S., Hombauer,M., Truppe,M., Vecsei,V. & Schlegel,W. Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes. *J. Bone Joint Surg. Br.* **86**, 286-295 (2004).

36. Marlovits,S., Hombauer,M., Tamandl,D., Vecsei,V. & Schlegel,W. Quantitative analysis of gene expression in human articular chondrocytes in monolayer culture. *Int. J. Mol. Med.* **13**, 281-287 (2004).

37. Buckwalter,J.A. & Mankin,H.J. Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr. Course Lect.* **47**, 477-486 (1998).

38. Gelse,K., Poschl,E. & Aigner,T. Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* **55**, 1531-1546 (2003).

39. Eyre,D. Collagen of articular cartilage. *Arthritis Res.* **4**, 30-35 (2002).

40. Buckwalter,J.A., Mankin,H.J. & Grodzinsky,A.J. Articular cartilage and osteoarthritis. *Instr. Course Lect.* **54**, 465-480 (2005).

41. Mankin,H.J. Chondrocyte transplantation--one answer to an old question. *N. Engl. J. Med.* **331**, 940-941 (1994).

CHAPTER FOUR

THE EFFECTS OF HIGH DENSITY CULTURE ON CHONDROCYTE PHENOTYPE

The effects of high density culture on chondrocyte genotype.

4.1 Introduction

Articular cartilage (AC) is an avascular tissue with poor healing potential following injury. The natural AC repair mechanisms result in the formation of sparse tissue with poor functional characteristics leading to eventual deterioration and osteoarthritis (OA) formation[1-6]. The challenge involved with development of AC repair techniques has turned out to be substantial with several different methods currently being employed[2,6]. Biological approaches are still viewed as holding the most promise for the formation of a functional and long lasting repair but much work is still required.

Arguably the current standard for focal AC defect repair is autologous chondrocyte implantation (ACI)[7]. This process involves the removal of a small portion of normal AC from a non-weight bearing region of the joint during an initial surgical procedure, harvest and expansion of the chondrocyte number *in vitro,* followed by implantation of the expanded chondrocyte population into the AC defect under cover of a membrane or with assistance of a matrix carrier[8-10]. Despite criticisms of this technique[2,11,12]cartilage repair procedures have been performed in this manner for over a decade with studies suggesting real patient benefit[7]. It is, however, recognized that improvements are required to make this technique more reliable, durable, and applicable to larger defects[13,14].

One of the problems associated with ACI is that it utilizes chondrocytes which have been expanded in monolayer culture resulting in loss of the differentiated chondrocyte phenotype[15-17]. This process, termed de-

differentiation, is characterized by the change in expression of matrix-forming collagen type II to the fibrocartilage-forming collagen type I[14]. In an attempt to overcome this problem, several culture systems have been developed which attempt to reverse this alteration in phenotype. This process, described as re-differentiation, is unfortunately unable to return the chondrocytes fully to their *in vivo* functional state once the chondrocytes have been cultured in monolayer. In particular, once mRNA for collagen type I is produced, no method appears to be able to completely eliminate it.

Much of the work attempting to restore *in situ* chondrocyte phenotype has involved passaging the cells three or more times and then modulating the cellular environment with techniques such as restoration of the rounded chondrocyte shape[18], medium supplementation with biomolecules[19], alteration in oxygen tension[20,21], or growth in high-density cultures[22]. Although groups have been successful in up-regulating collagen type II gene expression, they have been unable to eliminate the detrimental production of collagen type I which appears during culture[23-26]. It is this production of collagen type I which is thought to contribute to the formation of a *de novo* matrix that lacks the appropriate biomechanical properties and long term durability[2]. The poor integrity of this fibrocartilage repair tissue is likely due to the inability of collagen type I to interact and retain aggrecan in the same manner as the native collagen type II within the matrix[27].

Our previous work has demonstrated that collagen type I is rapidly up-regulated when the cells are released from their native matrix. In addition,

previous studies have been unable to fully reverse this up-regulation after the cells have been passaged suggesting that this may be a permanent genetic modification which occurs early in culture. This study attempted to prevent early phenotypic alterations affecting collagen type I expression by placing chondrocytes in high density cultures immediately following digestion. It was hypothesized that cells placed in a high density environment favoring a more rounded shape and increased cell-cell interactions would result in the preservation of collagen type II and an inhibition of collagen type I gene expression.

4.2 Methods

Normal human AC was collected from cadavers (Human Organ Procurement and Exchange; HOPE program) with permission from the University of Alberta Hospital ethics committee. Chondrocytes were harvested from each patient, plated in monolayer and either expanded through to passage 7 or left at confluence. Messenger RNA was extracted from the cells *in situ*, at each passage and weekly from the confluent cells and analyzed using real time PCR (RT-PCR). Collagen I protein expression was detected with immunofluorescence in the passage 0 cell population. The methods are detailed below.

4.2.1 Chondrocyte isolation and culture

Chondrocytes were obtained from normal human AC collected from the weight bearing region of the femoral condyles. Strips of full-thickness cartilage (~1cm wide) were removed from the subchondral bone in strips using a sterile

scalpel. The strips were digested for 6 hours at 37ºC in collagenase type 1A (Sigma C-9891) (1mg/ml) in serum free DMEM-F12 and 1 ml of the solution was utilized for each gram of cartilage. The chondrocytes were collected through centrifugation at 400xg for 5 minutes and the cell pellet was washed twice with serum-containing medium. Chondrocytes were then plated in monolayer and passaged (1:2) when approaching confluence. At each passage chondrocytes were released by trypsinizing with 0.25% Trypsin + EDTA (Lonza). Half of the cells were pelleted and stored at -80ºC to await analysis while the remainder were carried through to the next passage until passage 7 was completed. Simultaneously, chondrocytes were seeded at a density of 18 000cells/cm^2 on seven 60mm tissue culture dishes. These cells were allowed to grow *ab libitum* at high density and one plate from this group was harvested weekly and stored at -80ºC. All cells were grown at 37ºC and 5% CO_2 in Chondrocyte Growth Medium (CGM) (Lonza) which was changed every 3-4 days.

4.2.2 RNA isolation and real time polymerase chain reaction (RT-PCR)

RNA was extracted from the chondrocyte cell lines utilizing the SV Total RNA Isolation System (Promega) according to the manufacturer's specifications. RNA isolation from the cartilage tissue required that the tissue be ground into a powder in liquid nitrogen with the Spex® Sample Prep freezer mill (model 6770). The ground cartilage was processed with the Total RNA Fatty and Fibrous Tissue Pack (Bio-Rad) according to the manufacturer's specifications with the following modification. In the initial step, instead of 100mg, 50mg of tissue was added to 1

ml of PureZol® because this was found to significantly increase RNA yields. The purity and quantity of RNA was determined by usual means using the $OD_{260/280}$ ratio.

cDNA was generated from all samples with the Superscript III 1st-strand synthesis system (Invitrogen) according to the manufacturers specifications. The reagents for the RT-PCR were taken from the QuantiTect SYBR Green PCR kit (Qiagen). The primers utilized are recorded in Table 4.1. Quantification and analysis for each of the reactions was carried out using the MYiQ single-color real-time PCR detection system (BioRad). The PCR conditions were as follows: 94°C for 15 seconds, annealing (GAPDH and Aggrecan 60°C; Collagen I and II 58°C) for 20 seconds, 72°C for 10 seconds for 45 cycles. The comparative C_T method was utilized to provide a relative quantification of gene expression.

4.3 Results

4.3.1 Collagen Type I

Expression of collagen type I gene *in situ* was limited but in the time between chondrocyte digestion and harvest at passage 0 or week 1 there was a substantial increase in collagen I expression ($\sim 2^6$). This up-regulation of genetic expression was substantial and maintained through all subsequent passages. Although there was variability between patients with respect to the degree of genetic expression up-regulation, the pattern and trend was consistent for all iterations of the experiment in both the passaged and confluent cells (Figure 4.1). It is important to note that in the cells left at confluence the level of collagen type

I gene expression failed to return to the levels found to be expressed *in situ* which were undetectable.

4.3.2 Collagen Type II

In both the passaged cells and those left at confluence the relative gene expression of collagen type II was up-regulated following digestion prior to being harvested at passage 0 or week 1, similar to the values seen for collagen type I. However, this up-regulation was not consistent when comparing the passaged and confluent cells at all time points. In those cells which were passaged, the gene expression decreased following passage 0, was already below *in situ* levels by passage 4, and by passage 7 the level of collagen type II transcript had decreased even further (Figure 4.2).

In contrast, for those cells left at confluence a much different pattern of gene expression was observed following passage 0. In these cells collagen type II gene expression was up-regulated ($\sim 2^{10}$) but there was not the rapid decline to baseline levels like that observed in the cells which were passaged. In the cells at high density there was an overall increase in collagen type II gene expression and the level of expression was maintained above *in situ* levels at all time points and was significantly higher than the levels seen in the passaged cells by week 4/passage3 (Figure 4.2).

4.3.3 Aggrecan

The pattern of relative expression of aggrecan was much less variable. In both groups there was a gradual but progressive decrease in aggrecan gene expression from the levels found *in situ* through all time points (Figure 4.3).

4.4 Discussion

This study was designed to determine the effects of high density culture on chondrocyte genotype. This is an important issue because the most successful technique being employed currently to repair AC (ACI) involves implanting cultured chondrocytes into AC defects at high densities. It has become evident that chondrocytes quickly lose their genotype with passaging during culture. We have demonstrated that immediately upon release from the AC matrix, the chondrocyte population alters the ratio of production of collagen type I and II with collagen type I becoming the major product. It has been speculated that implantation of cultured chondrocytes at high densities during ACI results in re-differentiation of the implanted cells[8], but this work demonstrates that this may not be the case. Consequently, during ACI fibroblast-like cells are most likely being implanted and, not surprisingly, a matrix containing collagen type I is produced that can ultimately break down.

In this study, it was thought that by allowing the cells to be in close contact during culture, a microenvironment conducive to chondrocyte re-differentiation would be achieved early in culture with resultant genotype maintenance. This was accomplished with regards to levels of collagen type II

mRNA as noted by the initial increase that was maintained as long as the culture was maintained at confluence. Conversely, in the populations of cells that were simply passaged, a smaller initial increase in collagen type II mRNA levels occurred but this quickly decreased to levels below those found *in situ*. While these results were encouraging they were countered by the upregulation of collagen type I mRNA. Immediately following release from the AC matrix, the chondrocyte populations in both the passaged and confluence situations increased their levels of collagen type I mRNA expression and these increased levels were maintained throughout the experiment in a parallel pattern. While the results for the levels of mRNA for collagen type II were encouraging as an indicator for maintenance of normal chondrocyte genotype during confluence culture, the results related to mRNA levels of collagen type I were discouraging. Thus, collagen type I upregulation may represent the major obstacle facing chondrocyte culture in preparation for more successful transplantation at sites of AC injury.

One intriguing aspect of this study was the production pattern of collagen types I and II. There was an immediate increase in mRNA levels for both collagen types I and II among the cell populations experiencing passaging or remaining at confluence following release from the AC matrix. It is important to note that this increased level of collagen type I mRNA remained elevated but did not increase further as the experiment proceeded. In contrast, the mRNA levels for collagen type II in the same cell populations increased initially but then decreased although at different rates. It is interesting to note that this decrease in mRNA levels for collagen type II was not associated with an equivalent increase

in production of collagen type I which suggested that the cells did not switch from producing collagen type II to producing collagen type I. From this information, it is plausible that not all the cells behave the same after release from the AC matrix. It must be realized that measurements of mRNA levels represent populations of cells and not individual cells. Therefore, it is possible that some cells responded by starting to produce mRNA for collagen type I (the mRNA levels for collagen type I are at zero in normal AC) while other cells simply reduce their levels of mRNA production for collagen type II. There was not a simple switch from producing mRNA for collagen type II to producing mRNA for collagen type I or there should be a consistent relationship in relative values between the respective productions of mRNA shown in the results. This conclusion was supported by our previous research that used immunofluorescence to demonstrate that during passage 0, not all cells obtained from normal AC showed the presence of collagen type I and that its distribution differed widely within even the cells that stained positively. Of course it is also possible that some cells are producing mRNA for both collagen types I and II but it is also evident that not all the cells are producing mRNA for the unwanted collagen type I. This suggested that if the cells that produce mRNA for only collagen type II could be isolated from those that produce mRNA for collagen type I, then better success at maintaining the chondrocyte genotype might be achieved during culture resulting in more appropriate cells for implantation.

To date, a review of the literature has failed to identify any technique capable of completely eliminating production of mRNA for collagen type I

among cells demonstrating its presence. This is a major obstacle to increasing cell number through culture for the ACI technique. Either an appropriate method needs to be found or the production of mRNA for collagen type I needs to be prevented at the outset if successful repair techniques involving cultured cells is to be achieved.

Throughout this paper it has been difficult to describe adequately the cells under discussion. By definition a chondrocyte is a cell found in cartilage and in the context of this work, a chondrocyte is a cell found in AC. Initially, this definition seems to be far too simple to be useful in research especially as the genotype is so dynamic and responds quickly to changes in the microenvironment of the cell. This ability to change genotype so readily suggests that a more complex definition of a chondrocyte might be futile. The ever-changing genotype provides a moving target as far as a precise definition is concerned unless the parameters of the microenvironment are specified in very fine detail and all components of the genotype are included which is clearly impossible. Expressions such as ‘chondrocyte-like’ (implying production of mRNA for collagen type II) and ‘fibroblast-like’ (implying production of mRNA for collagen type I) are useful in a very limited sense but, perhaps, are misleading in relation to the overall picture of cell behavior. It may be more accurate that future descriptions of cell characteristics restrict themselves to accurate genotype parameters with a description of cell origin being captured under the expression of ‘chondrocyte’ as simply indicating the tissue from which it was obtained.

This work also highlighted other aspects of the current cartilage literature which warrant comment. The terms de-differentiation and re-differentiation have become ubiquitous in the chondrocyte literature, but can be misleading and could be viewed as misnomers. Alterations in genetic expression through modulation of the cellular environment early in culture are in some respects reversible and may not represent an actual change in differential state but instead an environmental modulation of chondrocyte phenotype[14]. However, this work demonstrated that some of the early changes seen may not be reversible (primarily the up-regulation of collagen type I) and may represent an actual change in cellular differentiation resulting in an inability to completely recover the *in situ* matrix-forming potential. It is also possible that both types of change may be occurring during culturing. More precise methods are required to identify any epigenetic changes that may be occurring as cells differentiate and distinguish these from the environmental modulation of chondrocyte phenotype[14].

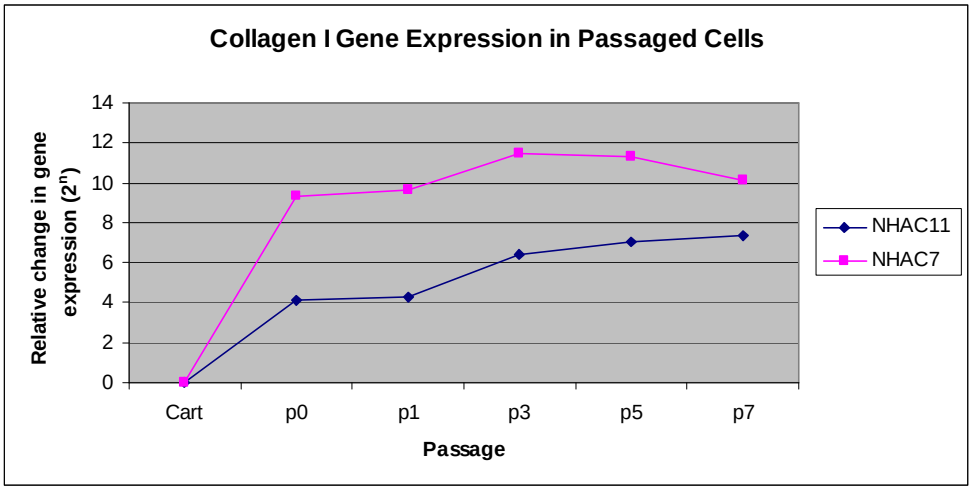
The effectiveness of a cartilage repair procedure is dependent upon the quality of the cells utilized for the repair. Because others have observed an up-regulation of collagen type II during attempts at chondrocyte re-differentiation, we have been deluded into believing that we are doing better than we really are. The results of this study have demonstrated that action needs to be taken early in chondrocyte harvest to prevent what appears to be an irreversible detrimental up-regulation of collagen type I. It is clear that further work is required to elucidate the mechanisms responsible for the early changes in gene expression including methods to monitor any epigenetic changes which may be occurring. In this way

we will be better equipped to generate chondrocytes which will form a more functional and longer lasting repair.

Gene	Size (bp)	Strand	Sequence(5´-3´)
Type II Collagen	257	**F**	GACAATCTGGCTCCCAAC
		R	ACAGTCTTGCCCCACTTAC
Type I Collagen	105	**F**	AGGTGCTGATGGCTCTCCT
		R	GGACCACTTTCACCCTTGT
Aggrecan	85	**F**	TCGAGGACAGCGAGGCC
		R	TCGAGGGTGTAGCGTGTAGAGA
GAPDH	189	**F**	TGGTATCGTGGAAGGACTCATGAC
		R	ATGCCAGTGAGCTTCCCGTTCAGC

Table 4.1. Primers for RT-PCR.

A)



B)

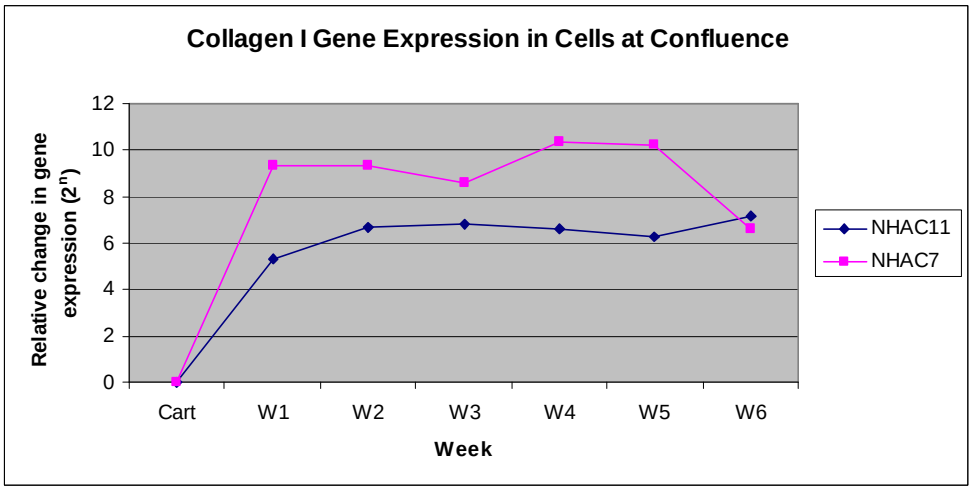
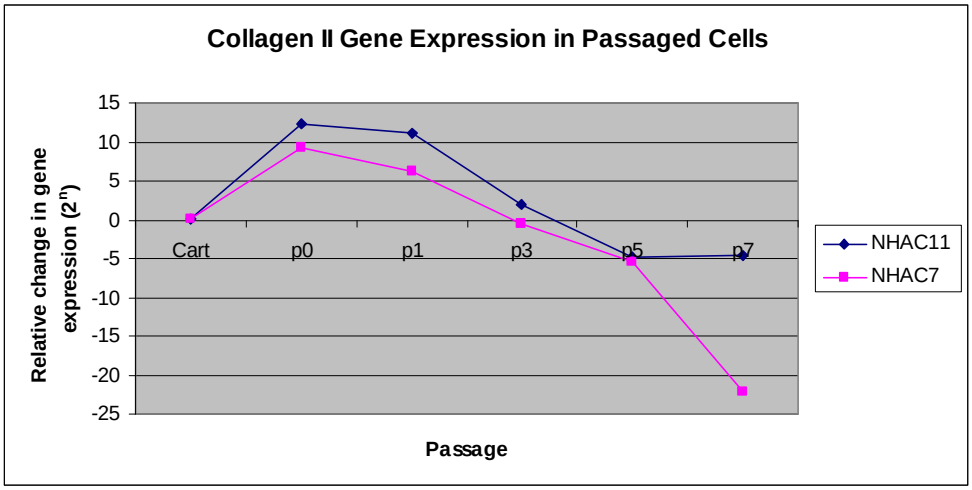


Figure 4.1. A) A graph demonstrating the change in collagen type I gene expression in passaged cells. Note that there is an immediate up-regulation in gene expression in passage 0 and that this is maintained in all passages. B) A graph demonstrating the expression of collagen type I in cells at confluence. Despite being maintained in a high density environment there is a pattern of collagen type I gene expression similar to the passaged cells.

A)



B)

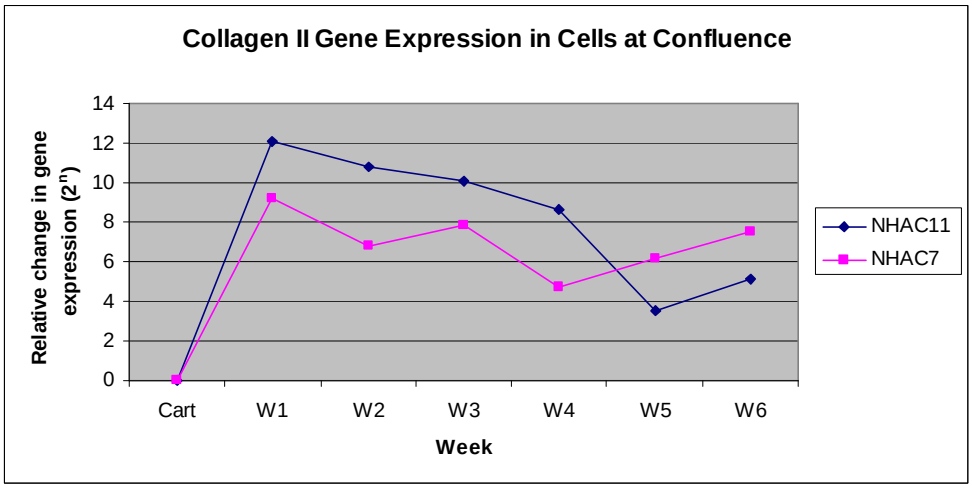
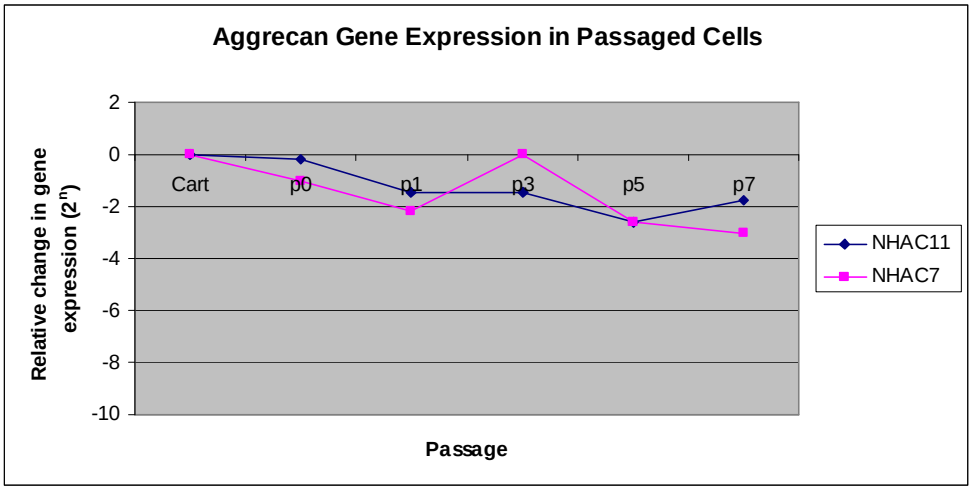


Figure 4.2. A) A graph outlining the gene expression of collagen type II in passaged chondrocytes. Note that there is an early increasing gene expression initially followed by a rapid down-regulation to baseline levels by passage 3. This trend continues and the expression is well below that found *in situ* by passage 7. B) A graph showing the gene expression of collagen type II in confluent cultures. Note that there is an up-regulation of collagen type II early as is seen in the passaged cells but in the high density cultures this increased expression is maintained above baseline at all time points.

A)



B)

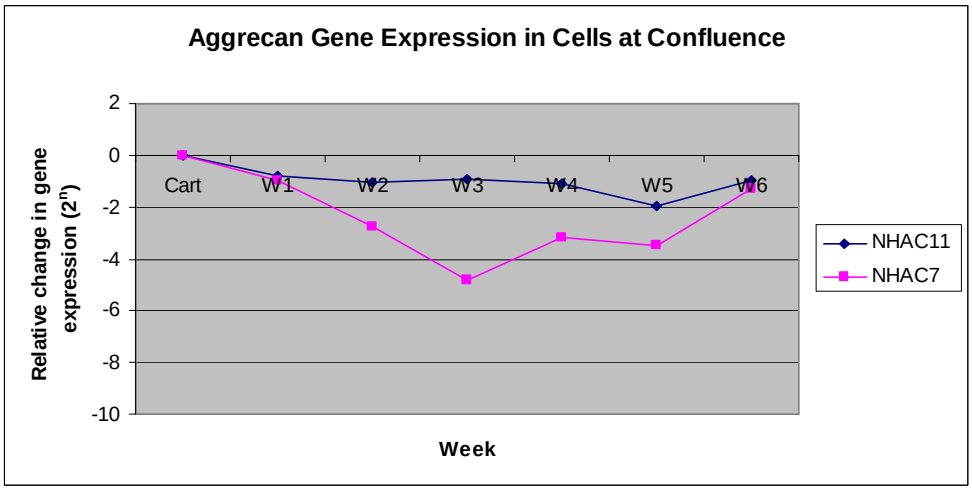


Figure 4.3. A) A graph showing the change in aggrecan gene expression in passaged chondrocytes. B) A graph showing the change in aggrecan gene expression in the cells left at confluence. Note that there is little difference between the two groups.

4.5 Reference List

1. Buckwalter,J., Simon,S. & Einhorn,T. Orthopedic Basic Science. Biology and Biomechanics of the Musculoskeletal System. 2nd Edition. (2000).

2. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage*. **10**, 432-463 (2002).

3. Buckwalter,J.A. & Martin,J.A. Sports and osteoarthritis. *Curr. Opin. Rheumatol.* **16**, 634-639 (2004).

4. Ritchie,P.K. & McCarty,E.C. Surgical management of cartilage defects in athletes. *Clin. Sports Med.* **24**, 163-174 (2005).

5. Meyer,U., Buchter,A., Wiesmann,H.P., Joos,U. & Jones,D.B. Current strategies for articular cartilage repair. *Eur. Cell Mater*. **9**, 39-49 (2005).

6. Alford,J.W. & Cole,B.J. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am. J. Sports Med.* **33**, 295-306 (2005).

7. Peterson,L., Brittberg,M., Kiviranta,I., Akerlund,E.L. & Lindahl,A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. *Am. J. Sports Med.* **30**, 2-12 (2002).

8. Brittberg,M., Peterson,L., Sjogren-Jansson,E., Tallheden,T. & Lindahl,A. Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments. *J Bone Joint Surg. Am.* **85-A Suppl 3**, 109-115 (2003).

9. Brittberg,M., Tallheden,T., Sjogren-Jansson,B., Lindahl,A. & Peterson,L. Autologous chondrocytes used for articular cartilage repair: an update. *Clin. Orthop. Relat Res.* S337-S348 (2001).

10. Lindahl,A., Brittberg,M. & Peterson,L. Cartilage repair with chondrocytes: clinical and cellular aspects. *Novartis. Found. Symp.* **249**, 175-186 (2003).

11. Driesang,I.M. & Hunziker,E.B. Delamination rates of tissue flaps used in articular cartilage repair. *J. Orthop. Res.* **18**, 909-911 (2000).

12. Grande,D.A., Pitman,M.I., Peterson,L., Menche,D. & Klein,M. The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. *J. Orthop. Res.* **7**, 208-218 (1989).

13. Yang,K.G. *et al.* Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. *Tissue Eng* **12**, 2435-2447 (2006).

14. Hardingham,T.E., Oldershaw,R.A. & Tew,S.R. Cartilage, SOX9 and Notch signals in chondrogenesis. *J Anat.* **209**, 469-480 (2006).

15. Benya,P.D., Padilla,S.R. & Nimni,M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313-1321 (1978).

16. Benya,P.D., Padilla,S.R. & Nimni,M.E. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. *Biochemistry* **16**, 865-872 (1977).

17. Cheung,H.S., Harvey,W., Benya,P.D. & Nimni,M.E. New collagen markers of 'derepression' synthesized by rabbit articular chondrocytes in culture. *Biochem. Biophys. Res. Commun.* **68**, 1371-1378 (1976).

18. Benya,P.D. & Shaffer,J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**, 215-224 (1982).

19. Francioli,S.E. *et al.* Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: Relevance for Automated Bioreactor Systems. *Tissue Eng* (2007).

20. Domm,C., Schunke,M., Steinhagen,J., Freitag,S. & Kurz,B. Influence of various alginate brands on the redifferentiation of dedifferentiated bovine articular chondrocytes in alginate bead culture under high and low oxygen tension. *Tissue Eng* **10**, 1796-1805 (2004).

21. Domm,C., Schunke,M., Christesen,K. & Kurz,B. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. *Osteoarthritis. Cartilage.* **10**, 13-22 (2002).

22. Schulze-Tanzil,G. *et al.* Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. *Cell Tissue Res.* **308**, 371-379 (2002).

23. Tallheden,T. *et al.* Gene expression during redifferentiation of human articular chondrocytes. *Osteoarthritis. Cartilage.* **12**, 525-535 (2004).

24. Barlic,A., Drobnic,M., Malicev,E. & Kregar-Velikonja,N. Quantitative analysis of gene expression in human articular chondrocytes assigned for autologous implantation. *J Orthop. Res.* (2008).

25. Darling,E.M. & Athanasiou,K.A. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop. Res.* **23**, 425-432 (2005).

26. Takahashi,T. *et al.* Three-dimensional microenvironments retain chondrocyte phenotypes during proliferation culture. *Tissue Eng* **13**, 1583-1592 (2007).

27. Gelse,K., Poschl,E. & Aigner,T. Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* **55**, 1531-1546 (2003).

CHAPTER FIVE

THE DEVELOPMENT OF OSTEOBLASTS FROM STEM CELLS TO SUPPLEMENT FUSION OF THE SPINE DURING SURGERY FOR AIS

The development of osteoblasts from stem cells to supplement fusion of the spine during surgery for AIS.

5.1 Introduction

Adolescent idiopathic scoliosis (AIS) is represented as an abnormal curvature of the spine with unknown aetiology. Most commonly, AIS occurs in children under the age of 16 years with an incidence of 1-2 % in the general population. Typically, the clinical course for AIS shows a progressive lateral curvature of the spine that becomes aggressive when the patient enters adolescence. Extreme cases (>45-50° Cobb angle) are usually treated by deformity correction, spinal instrumentation and bone fusion of the curved segment.

Successful surgical correction of AIS requires a stable bone fusion of the involved segmental spine to prevent progression of the deformity and improve the long-term prognosis for the patient. Iliac crest autograft has long been the “gold standard” for bone graft material. However, this graft is associated with increased morbidities and has important limitations. Morbidities include excessive blood loss, pain, scarring, and deformity at the harvest site. A major limitation is the amount of graft available. Since most patients with AIS are children and spinal deformation involves several segments of the spinal vertebrae, iliac crest autografts frequently provide insufficient bone for successful fusions. For these reasons, there is intense interest in developing alternative bone generating materials for spinal fusion to facilitate more rapid and robust bone fusion with less morbidity.

In recent years, tissue engineering approaches have been used to enhance bone healing and fusion. This has involved the use of synthetic matrices, supplementary cells and bioactive molecules or minerals [5,6,7,11,12,13,14,15]. Some of these biomaterials have been used successfully in the clinic for treatment of bone defects and as delivery systems for osteogenic substances.

Bone marrow-derived mesenchymal stem cells (BMSCs) appear to be an attractive source for autologous bone-forming cells in bone tissue engineering. BMSCs can be isolated from small volumes of aspirated bone marrow and expanded to a relatively large population *ex vivo* using cell culture techniques.[8] BMSCs represent a phenotypically and functionally heterogeneous population of mesenchymal stem cells which contribute to multiple lines of haemopoietic cells, bone, cartilage, adipocytes, myocytes and other cells in connective tissues[9,10]. It is anticipated that a combination of BMSC-derived osteogenic cells with appropriate biomaterials could prove a novel alternative to bone graft by providing osteoconduction and osteoinduction capabilities. The use of autologous BMSCs combined with synthetic biomaterials would overcome the immunogenecity of allogenic bone grafts and make this approach far more attractive for a wider clinical application.

While the ultimate goal is to develop a means by which BMSCs accompanied by an appropriate matrix can be introduced to the site of surgery during a spine fusion to aid in healing (for example, during instrumentation for AIS), this project focuses on the acquiring, isolation, expansion and characterization of BMSCs from femoral reaming as a first step.

5.2 Methods

5.2.1 Isolation and Expansion of hMSCs

Intramedullary reamings were collected from patients undergoing total hip arthroplasty. Cells were isolated from the reamings using a 100µm cell strainer (BD Falcon) in the presence of heparin. The bone chips were washed with warm PBS (Ca^{++} and Mg^{++} free) and the liquid phase of the wash was again passed through the cell strainer and collected. The bone chips were treated with 0.05% trypsin and 0.1% EDTA solution prepared in PBS and incubated for 5 minutes at 37°C. The suspension was passed through the cell strainer, the liquid portion collected and the bone chips discarded. Density gradient centrifugation utilizing Histopaque-1077 (Sigma) was employed to separate samples. The fraction at the plasma-Histopaque interface was collected. Viability was assessed and the cells were plated to a density of 6000 cells/cm^2 in monolayer culture with DMEM plus 10% FBS and 5ng/ml FGF2 at 37°C and 5% CO_2.

5.2.2 Immunophenotyping by Flow Cytometry

Cell surface antigens from passage 3 cells were analyzed with flow cytometry. Cells were fixed with cold 1% formaldehyde, washed with PBS, and then stained with the primary antibodies outlined in Table 5.1. The cell population was analyzed on the FACS Calibur (BD Biosciences, Mississauga, Ontario) using Cell Quest Pro software and compared to the isotype control.

5.2.3 MSC Differentiation

For osteogenic differentiation cells were allowed to become 70% confluent and treated with osteogenic selective medium (R&D Systems) according to the manufacturer's specifications. For adipogenic differentiation, cells were grown to 95% confluence and treated with adipogenic selective medium (R&D Systems). For chondrogenic differentiation cells were encapsulated in alginate beads .[16] The beads were placed in a 100mm culture dish with 15ml of Chondrocyte Differentiation Medium (CDM; Cambrex) at 37°C and 5% CO_2. The medium in all cases was changed every 3-4 days for 21days. Following differentiation the cells were plated in monolayer culture in CGM at 37°C and 5% CO_2 in preparation for subsequent analysis.

5.2.4 Immunofluorescence (IF) and Histology

IF for the adipogenic and osteogenic lines was carried out as per the manufacturer's protocol utilizing primary antibodies to fatty acid binding protein 4 (FABP4; R&D) and osteocalcin (R&D) respectively. For IF of the chondrogenic line, cells were fixed with 100% MeOH and blocked with 5% milk in PBS. Primary antibody consisting of either a 1:100 dilution of Anti-Collagen Type II IgG (Calbiochem) or a 1:25 dilution of Anti-hAggrecan (R&D Systems) was utilized. Secondary antibodies included a 1:100 dilution of CY-3 conjugated goat anti-rabbit IgG (Jackson Immunoresearch) and a 1:100 solution of Alexa Fluor anti-sheep IgG (Molecular Probes) for the collagen and aggrecan treatments respectively. Slides were mounted with aqueous mounting medium (R&D

Systems). Negative controls consisted of cell preparations incubated without primary antibody. For histological assessment, differentiated tissues were stained directly on the tissue culture surface. For the adipogenic line, Oil red O (ORO; Sigma) was employed to detect the intracellular lipid droplets .[17] The osteogenic line was stained for mineralization with Alizarin Red .[17] Osteogenic cells were then stained for alkaline phosphatase with the Sigma Alkaline Phosphatase Kit according to the manufacturers specifications.

5.2.5 RNA Isolation and Real Time Polymerase Chain Reaction (RT-PCR)

RNA was extracted from the chondrogenic cell line and the undifferentiated MSC control utilizing the SV Total RNA Isolation System (Promega). cDNA was generated with the Superscript III 1st-strand synthesis system (Invitrogen) according to the manufacturers specifications. The reagents for the RT-PCR were taken from the QuantiTect SYBR Green PCR kit (Qiagen). Primers utilized are recorded in Table 5.2. Quantification and analysis for each of the reactions was carried out utilizing the MYiQ single-color real-time PCR detection system (BioRad). The PCR conditions were as follows: 94°C for 15 seconds, annealing (GAPDH and Aggrecan 60°C; Collagen I and II 58°C) for 20 seconds, and 72°C for 10 seconds for 45 cycles.

5.3 Results

5.3.1 Isolation, Expansion and Characterization of Cell Isolates

Within 5 days following application of cells to the culture dishes, adherent fibroblast-like cells were observed and confluency was achieved by 14-21 days. Cells were detached with 0.25% trypsin-EDTA (Gibco) treatment, split 1:2 and in subsequent passages the cells rapidly became confluent within 7 days. Cell surface antigens on the adherent fibroblast-like cells were analyzed by flow cytometry. It was noted that these cells did not express the negative stem cell markers, CD45 or GlycoA, making a hematopoietic origin less likely. The cells did express CD13, CD29, CD44, CD54, CD90, and CD105 cell surface antigens, consistent with a MSC immunophenotype.

5.3.2 In Vitro Differentiation into Adipocytes, Osteocytes and Chondrocytes

Osteogenic differentiation was successful when attempted on passage 3 cells utilizing osteogenic selective medium. After 21 days the cells demonstrated a distinct increase in monolayer density, forming a lattice like appearance. Tissue harvested at this time demonstrated evidence of both ALP and mineralization as demonstrated by Alizarin Red staining. IF for osteocalcin was also positive.

Adipogenic differentiation was also performed on passage 3 cells using adipogenic selective medium. During differentiation, lipid droplets were evident after 7 days in a portion of the cells and this number increased as the cells were cultured up to 21 days. At this time the lipid inclusions stained positive with ORO and IF demonstrated the presence of FABP4 .

Chondrogenic differentiation was successful with MSCs at passage 3. Differentiated cells were removed from the three dimensional alginate environment and IF for collagen II in monolayer culture was positive while IF for aggrecan was only weakly positive. Real time PCR revealed an increase in Collagen II transcription compared to the pre-differentiated MSC population. There was negligible change in expression of GAPDH, a housekeeping gene, which was used as a standard. Both collagen I and aggrecan also demonstrated little change in expression when comparing the two cell populations. This was expected in the collagen I population but largely unexpected for aggrecan. This is likely due to the relatively short differentiation time of 3 weeks and it is hypothesized that as the differentiation time is increased increases in aggrecan upregulation will also be seen. The marked increase in collagen II production indicates expression of a chondrogenic phenotype.

5.4 Discussion

In the present study the *in vitro* isolation of MSCs from femoral intramedullary reamings collected intraoperatively is reported. Cells were separated using density gradient centrifugation and selected based on their ability to adhere in monolayer culture and rapidly expand in the presence of serum. These cells expressed the surface antigens CD13, CD29, CD44, CD54, CD90, and CD105 which are characteristic of a MSC immunophenotype. The cells did not possess the cell surface antigens CD45 and GlycoA which are characteristic of cells from a hematopoeic lineage. However, the immunophenotype alone is not

sufficient to define a MSC population. Therefore, the multilineage potential of the cell population through differentiation toward mesodermal lineages was established. Osteoblasts, adipocytes, and chondrocytes from passage 3 of the MSC population were successfully derived and their phenotypes confirmed with cell stains, fluorescent antibodies and RT-PCR.

Mesenchymal stem cells represent an easily acquired autologous cell source capable of being differentiated into a variety of different mesodermal lineages. The study demonstrates the ability to use femoral reamings to isolate, expand, and characterize successfully MSCs that can provide cells for future research. This is a necessary first step toward the final goal, which is the development of autologous osteogenic synthetic tissue to supplement spinal fusion.

Anti-Human	Dilution	Fluorochrome	Source
CD13	3/50	PE-Cy5	Caltag Laboratories
CD29	3/50	PE-CY5	Caltag Laboratories
CD44	1/10	Fluorosciene isothiocyanate (FITC)	Santa Cruz Biotechnologies
CD45	3/50	Phycoerythrin(PE)	Caltag Laboratories
CD54	1/50	FITC	Caltag Laboratories
CD90	1/50	PE	BD Biosciences
CD105	3/50	PE	Caltag Laboratories

Table 5.1. Antibodies for characterization of cell surface antigens.

Gene	Size (bp)	Strand	Sequence(5´-3´)
CollagenII	257	**F**	GACAATCTGGCTCCCAAC
		R	ACAGTCTTGCCCCACTTAC
Collagen I	105	**F**	AGGTGCTGATGGCTCTCCT
		R	GGACCACTTTCACCCTTGT
Aggrecan	85	**F**	TCGAGGACAGCGAGGCC
		R	TCGAGGGTGTAGCGTGTAGAGA
GAPDH	189	**F**	TGGTATCGTGGAAGGACTCATGAC
		R	ATGCCAGTGAGCTTCCCGTTCAGC

Table 5.2. Primers for RT-PCR..

5.5 References List

1. Mariconda M. Galasso O. Barca P. Milano C. Minimum 20-year follow-up results of Harrington rod fusion for idiopathic scoliosis. [Journal Article] European Spine Journal. 14(9):854-61, 2005 Nov.

2. Akbarnia BA. Marks DS. Boachie-Adjei O. Thompson AG. Asher MA. Dual growing rod technique for the treatment of progressive early-onset scoliosis: a multicenter study. [Journal Article. Multicenter Study] Spine. 30(17 Suppl):S46-57, 2005 Sep 1.

3. Kager AN. Marks M. Bastrom T. Newton PO. Morbidity of iliac crest bone graft harvesting in adolescent deformity surgery. [Journal Article] Journal of Pediatric Orthopedics. 26(1):132-4, 2006 Jan-Feb

4. Lewandrowski K. Bioresorbable bone graft substitutes of different osteoconductivities: *Biomaterials*. 2000; 21:757-764.

5. Wang P. Wang J. Bone graft substitutes for spinal fusion. The Spine Journal 2003; 3: 155 -165

6. Bruce T. Rougraff and Thomas J. Kling Treatment of Active Unicameral Bone Cysts with Percutaneous Injection of Demineralized Bone Matrix and Autogenous Bone Marrow J Bone Joint Surg Am 2002 84: 921-929.

7. Tiedeman JJ. Connolly JF. Strates BS. Lippiello L. Treatment of nonunion by percutaneous injection of bone marrow and demineralized bone matrix. An experimental study in dogs. Clinical Orthopaedics & Related Research. (268):294-302, 1991

8. Bianco P, Gehron Robey P. Marrow stromal stem cells. *J Clin Invest* 2000; 105: 1663–8.

9. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 1988; 136: 42–60.

10. Beresford JN. Osteogenic stem cells and the stromal system of bone and marrow. *Clin Orthop* 1989; 240:270–80)

11. Meinel L. Karageorgiou V. Hofmann S. Fajardo R. Synder B. Engineering bone-like tissue in vitro using human bone marrow stem cells and silk scaffolds. Journal of Biomedical Material Research 2005; 71A: 25-34

12. Frank O, Heim M, Jacob M, Barbero A, Schafer D, Bendik I, Dick W, Heberer M, Martin I. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. *J Cell Biochem* 2002; 85:737–46.

13. Sabokbar A, Millett PJ, Myer B, Rushton N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. *Bone Miner* 1994; 27:57–67.

14. Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R, Quarto R. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res* 2000; 49:328–37.

15. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue engineered bone regeneration. *Nat Biotechnol* 2000; 18: 959–63.

16. Ma,H.L.; Hung,S.C.; Lin,S.Y.; Chen,Y.L.; Lo,W.H. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. J.Biomed.Mater.Res.A 64(2) 273-281

16. Colter,D.C.; Sekiya,I.; Prockop,D.J. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc.Natl.Acad.Sci.U.S.A 98(14) 7841-45

CHAPTER SIX

ISOLATION OF A SUBPOPULATION OF MESENCHYMAL STEM CELLS WITH ENHANCED CHONDROGENIC POTENTIAL

Isolation of a subpopulation of mesenchymal stem cells with enhanced chondrogenic potential.

6.1 Introduction

The introduction of supplementary cells into a region of diseased or damaged tissue is becoming a viable treatment strategy in many areas of medicine. Indeed, tissue engineering techniques involving implantation of either autologous or allogenous cells along with supportive scaffolds are being widely investigated at the current time[1-3]. One area of tissue engineering which has generated much interest recently is the field of articular cartilage (AC) regeneration because damaged AC has and the subsequent osteoarthritis has such significant physical and economic impact. AC covers the ends of long bones forming diarthrodal joints and appears to be a simple tissue but in fact it possesses a complex set of biomechanical properties allowing it to resist large compressive forces while maintaining a nearly frictionless surface[4,5]. Unfortunately, AC also has a poor inherent healing capacity which results in an inability to form adequate repair tissue following injury[6,7]. This leads to progressive deterioration of the joint surface and the eventual formation of debilitating osteoarthritis (OA) [8-13]. Attempts to prevent this detrimental progression have led to the development of myriad different repair techniques[14-19] but none has proven completely successful in replicating the complex nature of AC and effecting a long term solution.

One technique of AC repair that is arguably the current standard is autologous chondrocyte implantation (ACI) which has been used for over a decade[20,21]. This technique involves the *ex vivo* expansion of chondrocytes harvested at an initial surgery followed by a second surgery to implant the

cultured cells beneath a periosteal patch or accompanied by a scaffold[22]. Although this technique has demonstrated some patient benefit, it is recognized that there is still much room for improvement[23,24]. For example, ACI requires two operative procedures and also must violate a normal area of AC to obtain the initial cartilage biopsy required for chondrocyte isolation. Perhaps more importantly, the *ex vivo*-expanded chondrocytes are known to lose their chondrocyte phenotype rapidly during monolayer culture resulting in the implantation of a population of fibroblast-like cells that produce predominantly collagen type I[25-27] rather than collagen type II which is the main constituent of the AC matrix. Perhaps it is not surprising that the resultant repair tissue lacks the necessary mechanical integrity and is prone to failure[28].

In an attempt to solve the deficiencies inherent to ACI, mesenchymal stem cells (MSCs) have been introduced as a viable alternative to mature chondrocytes for the repair of AC[29-39]. MSCs are particularly attractive because they represent an autologous, easily isolated, rapidly expandable, multipotent cell source with known chondrogenic capacity[40]. Their use would improve upon ACI because it would obviate the need for an initial arthrotomy and would also provide sufficient cell numbers because the MSCs would theoretically remain capable of differentiating towards the chondrogenic phonotype despite multiple cell divisions.

Unfortunately, MSCs are also not without their shortcomings. MSCs represent a heterogeneous and dynamic population of cells, comprised of a mixture of multi- and bi-potent progenitors, lineage restricted precursors, and

fibroblasts[41] and it is doubtful that each will function in the same manner. In an attempt to enhance the therapeutic potential of the MSC population, others[42,43] have tried to identify subpopulations of cells based on specific biological properties within the mixed MSC population. Although these groups have been successful in identifying a subpopulation of cells which appear to have enhanced neurogenic capabilities, no group to date has successfully identified a relationship between a MSC subpopulation and the multipotent progenitors responsible for generating cartilage. Towards this end this study isolated a subpopulation of MSCs displaying markers considered to enhance their chondrogenic potential. It was thought that by generating a more uniform population of cells the potential for AC tissue repair would be improved.

The marker selected to isolate the subpopulation of cells in this study was the CD44 cell surface antigen which is a single pass membrane glycoprotein expressed by chondrocytes and binds to hyaluronin in the AC extracellular matrix[44]. It is an important mediator in cell-matrix interactions and plays a vital role in cartilage matrix assembly and homeostasis[45-47]. While CD44 has also been identified on other cells and is used as a general marker for MSCs[48,49], it is seldom present on all of the cells in the mixed MSC milieu. Based on this information it was postulated that a purified subpopulation of MSCs, isolated based on the CD44 cell surface antigen, would have enhanced chondrogenic capacity when compared to the native heterogeneous MSC population. This would represent an important first step in identifying a better population of cells for implantation during AC repair surgery.

6.2 Methods

6.2.1 Isolation and Expansion of MSCs

Marrow aspirates were collected from patients undergoing harvest of iliac crest bone graft. A large-bore angio-catheter was used to collect the marrow and 1000 Units of heparin was added to the syringe for every milliliter of aspirate. In the laboratory, 3ml of the marrow suspension was layered over 3ml of Histopaque-1077 (Sigma) in a 15ml conical tube and centrifuged at 400*g* for 30 minutes. The fraction at the plasma-Histopaque interface was collected, washed twice with PBS, and plated in monolayer culture with Mesenchymal Stem Cell Growth Medium (MSCGM) (Lonza) at 37°C and 5% CO_2. After 48 hours in culture the non-adherent cells were removed and the remainder of the cells were passaged 1:2 when approaching confluence until the end of passage 5 was reached.

6.2.2 Immunophenotyping by Flow Cytometry

To determine the phenotype of the cell monolayer, the distribution of cell surface antigens from passage 5 cells was analyzed with flow cytometry. Cells were fixed with cold 1% formaldehyde (BDH Laboratory Supplies) and washed once with PBS. They were labelled with the primary antibodies outlined in Table 6.1. The cell population was analyzed on the FACS Calibur (BD Biosciences, Mississauga, Ontario) using Cell Quest Pro software and compared to the isotype control.

6.2.3 Cell Separation

Following trypsinization the passage 5 MSCs were washed twice in PBS and then resuspended in PBS + 2mM EDTA at a final concentration of $2.5X10^6$ cells/ml. The cells were labelled with the CD44-FITC conjugated antibody (Santa Cruz Biotechnologies) which was utilized at a 1:10 dilution. The labelled MSCs were sorted using Fluorescence-Activated Cell Sorting (FACS) (Beckman Coulter EPICS Ultra-High Speed Cell Sorter), generating two experimental populations of cells, one being CD44+ and the other CD44-. A separate aliquot of MSCs was subjected to the same treatment as outlined above but was not sorted using FACS, thereby generating a third group that was essentially the native mixed MSC population which acted as a normal control.

6.2.4 Chondrogenic Differentiation

Each of the three groups of cells, (CD44+, CD44- and the mixed population) were differentiated towards the chondrogenic lineage using previously outlined techniques[50]. Briefly, aliquots of each of the MSC populations (5.0X105 cells in 100µl of medium) were pipetted onto dry 6.5mm, 0.4µm pore size transwell membranes and spun in a 24-well plate centrifuge (200*g* for 5 min.). Culture was then carried out in a 24-well plate with 0.5ml of MSC Chondrogenic Differentiation Medium (Lonza) with 10µg/ml TGFβ3 added to the lower well which submerged the membranes. Medium was change every 2 days and the differentiation was carried out at 37°C and 5% CO_2. In each group differentiations were carried out in triplicate.

6.2.5 Adipogenic and Osteogenic Differentiation and Staining

Adipogenic and osteogenic differentiation was also performed to confirm the multipotent nature of the three MSC populations. For osteogenic differentiation, the cells were allowed to become 70% confluent and treated with osteogenic selective medium (R&D Systems) according to the manufacturer's specifications. For adipogenic differentiation, cells were grown to 95% confluence and treated with adipogenic selective medium (R&D Systems). In both protocols the medium was changed every 3-4 days for 21 days. Immunofluorescence for the adipogenic and osteogenic lines was performed as per the manufacturer's protocol utilizing primary antibodies to fatty acid binding protein 4 (FABP4) (R&D Systems) and osteocalcin (R&D Systems) respectively. Negative controls included cell preparations which had been incubated without primary antibody. Slides were viewed using fluorescence microscopy and digital images were collected for analysis.

6.2.6 RNA Isolation and Real Time Polymerase Chain Reaction (RT-PCR)

RNA was extracted from the three differentiated MSC-chondrogenic cell lines and the undifferentiated MSC population utilizing the SV Total RNA Isolation System (Promega). cDNA was generated with the Superscript III 1st-strand synthesis system (Invitrogen) according to the manufacturers specifications. The reagents for the RT-PCR were taken from the QuantiTect SYBR Green PCR kit (Qiagen). Primers utilized are recorded in Table 6.2.

Quantification and analysis for each of the reactions was carried out utilizing the MYiQ single-color real-time PCR detection system (BioRad). The PCR conditions were as follows: 94°C for 15 seconds, annealing (GAPDH and Aggrecan 60°C; Collagen I and II 58°C) for 20 seconds, 72°C for 10 seconds for 45 cycles. All PCR was performed in quadruplicate. The comparative C_T method was utilized to provide a relative quantification of gene expression with the undifferentiated MSC population acting as the baseline.

6.2.7 Statistical analysis

The experiment was performed in triplicate and data were compared within each group and between groups using one way ANOVA and the software package SPSS.

6.3 Results

6.3.1 MSC Isolation, Immunophenotyping and Cell Separation

A population of cells was isolated which was adherent to the tissue culture surface and expanded rapidly to fill the plates. Flow cytometry revealed that the isolated cell population possessed an immunophenotype consistent with other previously established human MSC populations (Figure 6.1). FACS was able separate the CD44+ and CD44- populations. Of those cells that stained positively for CD44, only the 82.1% that fluoresced most intensely were included in the CD44+ population and used for subsequent analysis.

6.3.2 Osteogenic and Adipogenic Differentiation

Cells in each of the three experimental groups differentiated towards the osteogenic and adipogenic lineages (as well as chondrocytes), confirming multipotent ability (Figure 6.2). In each of the three separate groups of cells there was no apparent difference in pattern or number of differentiated osteogenic and adipogenic cells.

6.3.3 Chondrogenic Differentiation and Real Time PCR Analysis

Real time PCR carried out on the CD44+, CD44- and mixed cell populations revealed that the relative level of collagen type II gene expression increased in all groups. However, statistical significance was only seen when comparing the relative increases in the CD44+ and the mixed cell population (Figure 6.3) where it was found that the level of collagen type II gene expression was significantly higher in the CD44+ population ($p=0.006$). The difference in the increase of collagen type II gene expression in CD44- population did not reach statistical significance when compared to either the CD44+ ($p=0.690$) or the mixed populations ($p=0.330$).

The average increase in aggrecan gene expression followed a pattern similar to that seen in collagen type II. An increase was seen in all three experimental populations but statistical significance was only seen when comparing the CD44+ and the mixed population results (Figure 6.4) where there was more aggrecan gene expression seen in the CD44+ population ($p=0.020$). There was no statistical significance reached when comparing the CD44-

population to either the CD44+ population (p=0.580) or the mixed population (p=0.183).

The relative levels of increase in collagen type I gene expression also increased approximately 6 fold in all groups (Figure 6.5) with no significant difference being found between any of these values.

6.4 Discussion

Despite several decades of study and their potential for widespread therapeutic use, the exact nature of MSCs has not been well defined. The basic biological properties of this mixed population are not well characterized although attempts have been made to identify different subpopulations of MSCs based on classes of regulatory proteins[51]. Notwithstanding these recent advances and great interest in the role of MSCs in cartilage regeneration, to date no group has been able to identify any subpopulation of progenitors responsible for differentiation towards the chondrogenic lineage. With this in mind, the CD44 cell surface protein was selected for use in this study because it is present on the surface of chondrocytes and has a specific role in binding to hyaluronin within AC which is important in AC matrix generation and homeostasis. CD44 is also known to be present on a portion of the cells within the general MSC population and was therefore selected as a target regulatory protein which may have the potential to identify those cells with greater chondrogenic abilities.

This study demonstrated that there were differences in gene expression when comparing a CD44+ purified subpopulation of MSCs and the native, mixed

MSC population following chondrogenic differentiation. The CD44+ population displayed a larger relative increase in both the collagen type II and aggrecan gene expressions. These proteins are the predominant components of the AC matrix and are essential if new hyaline cartilage is to be generated. While these results represent a promising first step towards our ultimate goal of providing more appropriate cells for implantation they need to be approached with caution. Although these differences were found to be statistically significant, the differences were quite small (~1 fold) and were obtained using a small sample size. As such, they may not represent a clinically significant difference in matrix production if these cells were placed in the appropriate environment and allowed to generate a *de novo* matrix.

It is also important to note that the levels of collagen type I gene expression were increased in all three populations of cells explored when compared to the undifferentiated MSC population. The presence of collagen type I represents a significant hurdle which must be surpassed because it is not a component of normal AC. Indeed, one of the arguments against using mature chondrocytes for AC tissue engineering is that they rapidly loose their phenotype, producing increased amounts of collagen type I, leading to formation of non-durable fibrocartilage. In fact, the gene expression profiles observed by the MSC-derived chondrocytes in this study resemble closely those of dedifferentiated early passage chondrocytes observed in previous work. This again leads to questions regarding the nature and definition of a chondrocyte.

Traditionally, a combination of the production of collagen type II and an absence of collagen type I has loosely defined an articular chondrocyte once it is removed from its native matrix[52]. If this definition is utilized, the cells generated in this study, even those in the CD44+ population, could only be viewed as chondrocyte-like at best because they produce both collagen type I and collagen type II. Further work is certainly required to further elucidate the mechanisms responsible for the unwanted genetic upregulation of the collagen type I gene and ways to decrease its expression. Additionally, the use of the term chondrocyte when describing cells simply on the basis of collagen type II production needs to be reconsidered.

It is also possible that the subpopulations studied were not sufficiently purified and still represent heterogeneous cell populations to some extent. This could explain the consistent collagen type I gene expression observed in all limbs of the experiment while there was still variability in the expression of collagen type II and aggrecan. It is possible that a group of cells responsible for producing the majority of the collagen type I was not removed during the sorting process and were present in all of the experimental subpopulations. This stresses the need for additional markers in order to further purify the MSC population to achieve a more chondrocyte-like cell line in the future.

There are those who may speculate that purification of the MSC population is not required and may be a lesson in futility. It may be said that these cells are in a constant state of flux and sufficiently plastic that they can rapidly change their regulatory proteins and subsequently their phenotype in

response to their environment despite being purified. Indeed, groups have demonstrated that different isolation schemes do induce a variety of epigenetic and genetic changes[53]. Unfortunately these changes are just as likely to limit plasticity and greatly affect the cells' therapeutic potential. It is for these reasons that standardized isolation and culture techniques need to be established if we hope to ever compare and reproduce experimental results. This will enable us to continue to better isolate and characterize MSC subpopulations which will greatly improve their disease-specific potential and enhance their therapeutic effect.

Anti-Human	Dilution	Fluorochrome	Source
CD13	3/50	PE-Cy5	Caltag Laboratories
CD29	3/50	PE-CY5	Caltag Laboratories
CD44	1/10	Fluorosciene isothiocyanate (FITC)	Santa Cruz Biotechnologies
CD34	1/50	FITC	BD Biosciences
CD45	3/50	Phycoerythrin(PE)	Caltag Laboratories
CD90	1/50	PE	BD Biosciences
CD105	3/50	PE	Caltag Laboratories

Table 6.1. Antibodies for characterization of cell surface antigens.

Gene	Size (bp)	Strand	Sequence(5´-3´)
Collagen II	257	**F**	GACAATCTGGCTCCCAAC
		R	ACAGTCTTGCCCCACTTAC
Collagen I	105	**F**	AGGTGCTGATGGCTCTCCT
		R	GGACCACTTTCACCCTTGT
Aggrecan	85	**F**	TCGAGGACAGCGAGGCC
		R	TCGAGGGTGTAGCGTGTAGAGA
GAPDH	189	**F**	TGGTATCGTGGAAGGACTCATGAC
		R	ATGCCAGTGAGCTTCCCGTTCAGC

Table 6.2. Primers for RT-PCR.

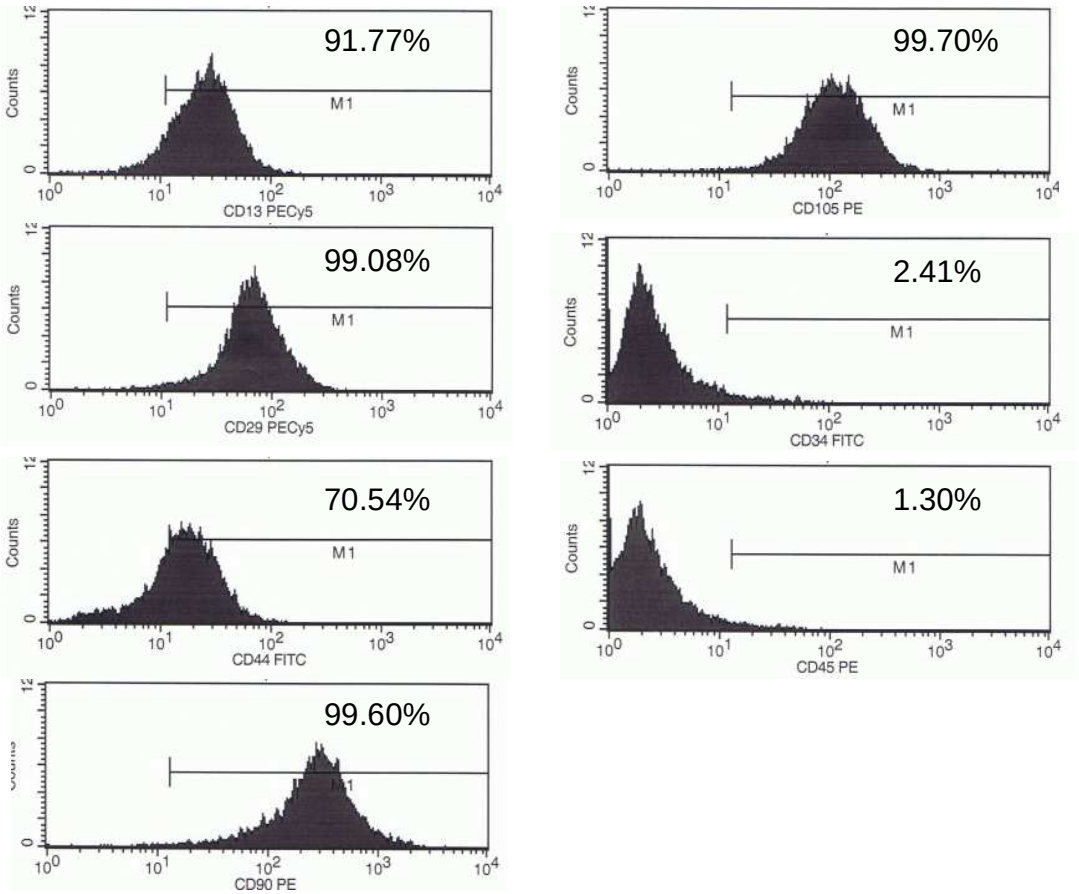


Figure 6.1. Flow cytometry results for the MSC population. The population did not possess the CD45 and CD34 cell surface antigens as would be expected. The population was positive for the other antigens which are considered positive markers of MSCs. Note that not all of the cells in the population were positive for CD44.

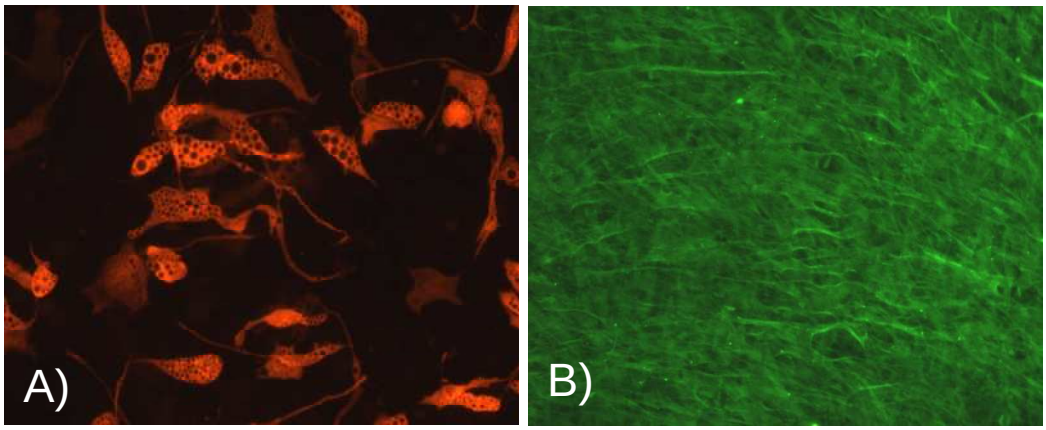


Figure 6.2. A) Representative fluorescence micrograph demonstrating staining for FABP4 within the cytoplasm of the MSC-differentiated adipocytes. B) Representative fluorescence micrograph demonstrating extracellular staining for osteoacalcin in the MSC-differentiated osteocytes.

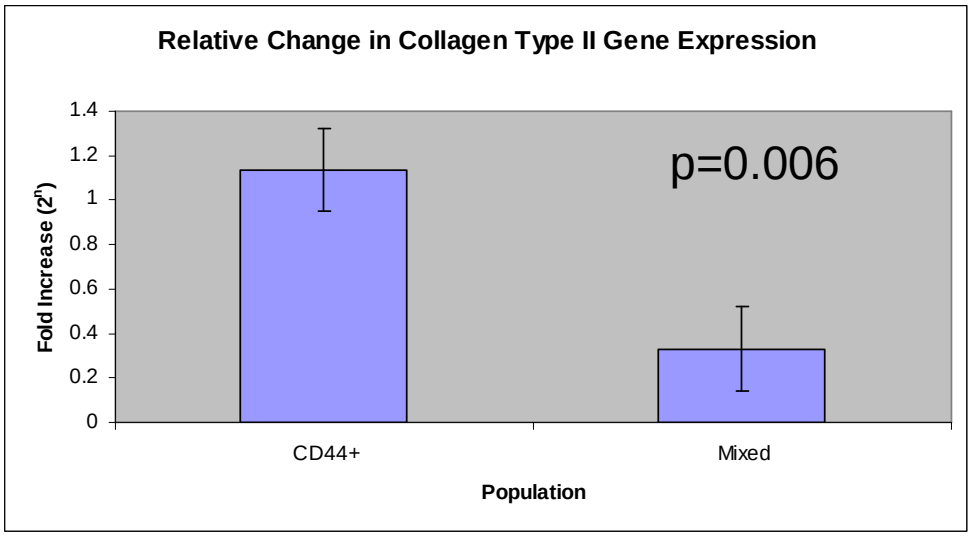


Figure 6.3. Graph comparing the relative difference in collagen type II gene expression between the CD44+ subpopulation and mixed cell population.

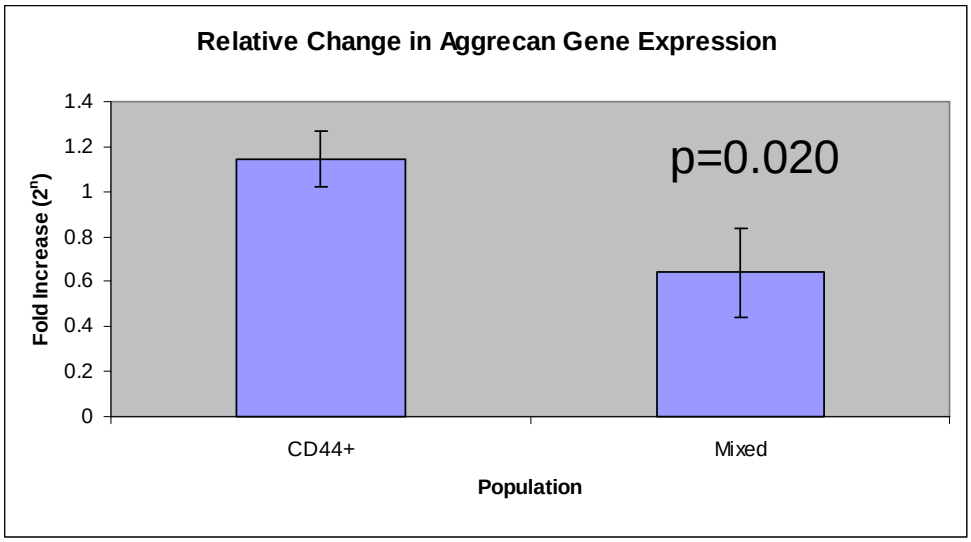


Figure 6.4. Graph comparing the relative difference in aggrecan gene expression between the CD44+ subpopulation and mixed cell population.

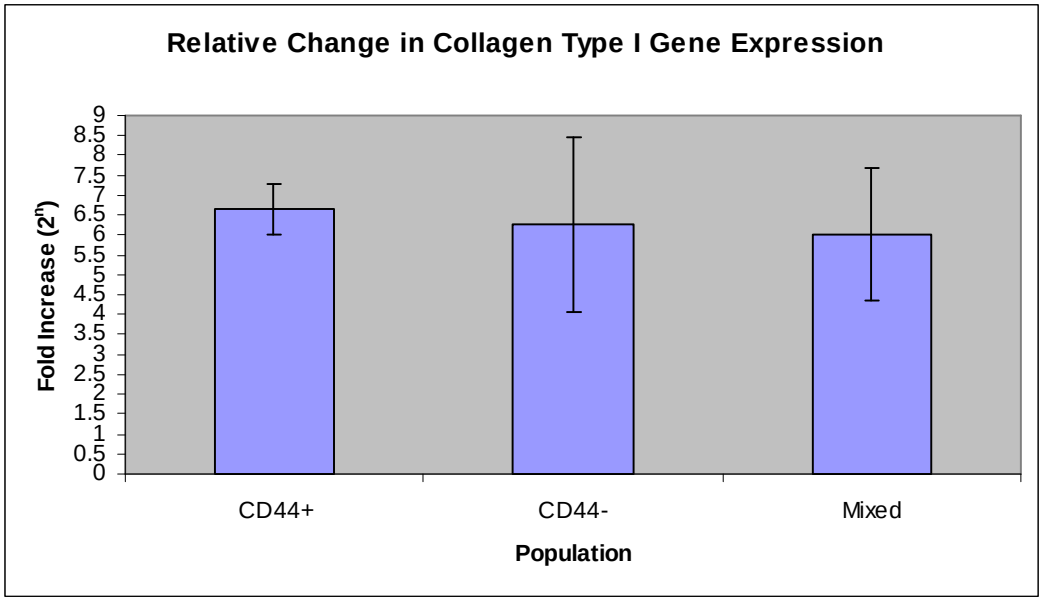


Figure 6.5. Graph comparing collagen type I gene expression in the three experimental groups. Note that there was a similar increase in all three groups and that there were no statistically significant differences between the groups.

6.5 Reference List

1. Bobis,S., Jarocha,D. & Majka,M. Mesenchymal stem cells: characteristics and clinical applications. *Folia Histochem. Cytobiol.* **44**, 215-230 (2006).

2. Schuleri,K.H., Boyle,A.J. & Hare,J.M. Mesenchymal stem cells for cardiac regenerative therapy. *Handb. Exp. Pharmacol.* 195-218 (2007).

3. Phinney,D.G. & Prockop,D.J. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* **25**, 2896-2902 (2007).

4. Buckwalter,J., Simon,S. & Einhorn,T. Orthopedic Basic Science. Biology and Biomechanics of the Musculoskeletal System. 2nd Edition. (2000).

5. Buckwalter,J.A., Mankin,H.J. & Grodzinsky,A.J. Articular cartilage and osteoarthritis. *Instr. Course Lect.* **54**, 465-480 (2005).

6. Buckwalter,J.A. Articular cartilage injuries. *Clin. Orthop. Relat Res.* 21-37 (2002).

7. Buckwalter,J.A. Sports, joint injury, and posttraumatic osteoarthritis. *J. Orthop. Sports Phys. Ther.* **33**, 578-588 (2003).

8. Buckwalter,J., Simon,S. & Einhorn,T. Orthopedic Basic Science. Biology and Biomechanics of the Musculoskeletal System. 2nd Edition. (2000).

9. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage.* **10**, 432-463 (2002).

10. Buckwalter,J.A. & Martin,J.A. Sports and osteoarthritis. *Curr. Opin. Rheumatol.* **16**, 634-639 (2004).

11. Ritchie,P.K. & McCarty,E.C. Surgical management of cartilage defects in athletes. *Clin. Sports Med.* **24**, 163-174 (2005).

12. Meyer,U., Buchter,A., Wiesmann,H.P., Joos,U. & Jones,D.B. Current strategies for articular cartilage repair. *Eur. Cell Mater.* **9**, 39-49 (2005).

13. Alford,J.W. & Cole,B.J. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am. J. Sports Med.* **33**, 295-306 (2005).

14. Alford,J.W. & Cole,B.J. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am. J. Sports Med.* **33**, 295-306 (2005).

15. Aubin,P.P., Cheah,H.K., Davis,A.M. & Gross,A.E. Long-term followup of fresh femoral osteochondral allografts for posttraumatic knee defects. *Clin. Orthop. Relat Res.* S318-S327 (2001).

16. Bartlett,W. *et al.* Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. *J. Bone Joint Surg. Br.* **87**, 640-645 (2005).

17. Beaver,R.J. *et al.* Fresh osteochondral allografts for post-traumatic defects in the knee. A survivorship analysis. *J. Bone Joint Surg. Br.* **74**, 105-110 (1992).

18. Behrens,P., Bitter,T., Kurz,B. & Russlies,M. Matrix-associated autologous chondrocyte transplantation/implantation (MACT/MACI)--5-year follow-up. *Knee.* **13**, 194-202 (2006).

19. Bentley,G. & Minas,T. Treating joint damage in young people. *BMJ* **320**, 1585-1588 (2000).

20. Brittberg,M., Peterson,L., Sjogren-Jansson,E., Tallheden,T. & Lindahl,A. Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments. *J Bone Joint Surg. Am.* **85-A Suppl 3**, 109-115 (2003).

21. Brittberg,M., Tallheden,T., Sjogren-Jansson,B., Lindahl,A. & Peterson,L. Autologous chondrocytes used for articular cartilage repair: an update. *Clin. Orthop. Relat Res.* S337-S348 (2001).

22. Bartlett,W. *et al.* Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. *J. Bone Joint Surg. Br.* **87**, 640-645 (2005).

23. Yang,K.G. *et al.* Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. *Tissue Eng* **12**, 2435-2447 (2006).

24. Hardingham,T.E., Oldershaw,R.A. & Tew,S.R. Cartilage, SOX9 and Notch signals in chondrogenesis. *J Anat.* **209**, 469-480 (2006).

25. Benya,P.D., Padilla,S.R. & Nimni,M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313-1321 (1978).

26. Benya,P.D., Padilla,S.R. & Nimni,M.E. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not

type II. Verifications by cyanogen bromide peptide analysis. *Biochemistry* **16**, 865-872 (1977).

27. Cheung,H.S., Harvey,W., Benya,P.D. & Nimni,M.E. New collagen markers of 'derepression' synthesized by rabbit articular chondrocytes in culture. *Biochem. Biophys. Res. Commun.* **68**, 1371-1378 (1976).

28. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage*. **10**, 432-463 (2002).

29. Cancedda,R., Dozin,B., Giannoni,P. & Quarto,R. Tissue engineering and cell therapy of cartilage and bone. *Matrix Biol.* **22**, 81-91 (2003).

30. Grande,D.A., Mason,J., Light,E. & Dines,D. Stem cells as platforms for delivery of genes to enhance cartilage repair. *J. Bone Joint Surg. Am.* **85-A Suppl 2**, 111-116 (2003).

31. Huang,C.Y., Reuben,P.M., D'Ippolito,G., Schiller,P.C. & Cheung,H.S. Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* **278**, 428-436 (2004).

32. Johnstone,B., Hering,T.M., Caplan,A.I., Goldberg,V.M. & Yoo,J.U. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* **238**, 265-272 (1998).

33. Kavalkovich,K.W., Boynton,R.E., Murphy,J.M. & Barry,F. Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system. *In Vitro Cell Dev. Biol. Anim* **38**, 457-466 (2002).

34. Ma,H.L., Hung,S.C., Lin,S.Y., Chen,Y.L. & Lo,W.H. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. *J. Biomed. Mater. Res. A* **64**, 273-281 (2003).

35. Noth,U., Tuli,R., Osyczka,A.M., Danielson,K.G. & Tuan,R.S. In vitro engineered cartilage constructs produced by press-coating biodegradable polymer with human mesenchymal stem cells. *Tissue Eng* **8**, 131-144 (2002).

36. Sekiya,I., Vuoristo,J.T., Larson,B.L. & Prockop,D.J. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proc. Natl. Acad. Sci. U. S. A* **99**, 4397-4402 (2002).

37. Stock,U.A. & Vacanti,J.P. Tissue engineering: current state and prospects. *Annu. Rev. Med.* **52**, 443-451 (2001).

38. Wang,Y., Kim,U.J., Blasioli,D.J., Kim,H.J. & Kaplan,D.L. In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. *Biomaterials* **26**, 7082-7094 (2005).

39. Williams,C.G. *et al.* In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. *Tissue Eng* **9**, 679-688 (2003).

40. Baksh,D., Song,L. & Tuan,R.S. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J. Cell Mol. Med.* **8**, 301-316 (2004).

41. Phinney,D.G. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle* **6**, 2884-2889 (2007).

42. Phinney,D.G. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle* **6**, 2884-2889 (2007).

43. Phinney,D.G. & Prockop,D.J. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* **25**, 2896-2902 (2007).

44. Knudson,W. & Loeser,R.F. CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell Mol. Life Sci.* **59**, 36-44 (2002).

45. Knudson,W. & Loeser,R.F. CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell Mol. Life Sci.* **59**, 36-44 (2002).

46. Knudson,C.B. & Knudson,W. Hyaluronan and CD44: modulators of chondrocyte metabolism. *Clin. Orthop. Relat Res.* S152-S162 (2004).

47. Knudson,C.B. Hyaluronan and CD44: strategic players for cell-matrix interactions during chondrogenesis and matrix assembly. *Birth Defects Res. C. Embryo. Today* **69**, 174-196 (2003).

48. Ishii,M. *et al.* Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts. *Biochem. Biophys. Res. Commun.* **332**, 297-303 (2005).

49. Gonzalez,R. *et al.* Pluripotent marker expression and differentiation of human second trimester Mesenchymal Stem Cells. *Biochem. Biophys. Res. Commun.* **362**, 491-497 (2007).

50. Murdoch,A.D. *et al.* Chondrogenic differentiation of human bone marrow stem cells in transwell cultures: generation of scaffold-free cartilage. *Stem Cells* **25**, 2786-2796 (2007).

51. Phinney,D.G. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle* **6**, 2884-2889 (2007).

52. Hardingham,T.E., Oldershaw,R.A. & Tew,S.R. Cartilage, SOX9 and Notch signals in chondrogenesis. *J Anat.* **209**, 469-480 (2006).

53. Phinney,D.G. & Prockop,D.J. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* **25**, 2896-2902 (2007).

CHAPTER SEVEN

CONCLUSION

7.1 Conclusion

OA is a disease which is ubiquitous in all populations and has significant individual, social, and economic impact worldwide[1-4]. Although there are many etiologies leading to joint deterioration and the eventual development of OA, one potentially treatable cause is the acute articular cartilage injury[5-7]. These injuries are common and have a poor inherent healing capacity[8,9]. Once a significant AC defect (>1cm) is generated, abnormal cartilage loading occurs, resulting in progressive cartilage loss such that eventually large areas of full thickness cartilage damage occurs. In an effort to repair AC injuries and preserve joint function many different treatment strategies have been developed but none have proven to be completely successful[10].

Studies of tissue-engineering strategies suggest that the most potential for success involves introduction of autogenous or allogenous cells to the site of injury, either alone or embedded within a supportive scaffold. These strategies are designed to encourage creation of a new matrix with the appropriate characteristics of normal AC which would be able to incorporate into the adjacent native tissue[11-13]. However, development of a successful repair method has proven difficult. The complex structural and biomechanical properties of normal AC are not easy to replicate, an expandable cell source with the appropriate functional characteristics has not been identified, and the problem of effective incorporation of a repair construct into the host tissue remains unresolved.

In an effort to overcome these obstacles and more fully understand the cartilage repair process, this work first focused on the development and utilization

of an *in vitro* human explant model of AC to study the ability of seeded human chondrocytes to adhere and integrate into an AC defect. A human derived model was employed because animal models have many features which differ significantly from the human which may affect results. A reproducible *in vitro* model for AC injury was successfully developed and found to be viable for up to 28 days in culture. Following seeding of allogenous chondrocytes into these explant defects it was noted that the cells became adherent to injured areas, both in the experimental defects and also in regions where there was damage to the lamina splendens. Once adherent, the seeded chondrocytes produced a matrix containing collagen II but unfortunately the matrix formed was too sparse for this type of technique to be considered feasible. It was also noted that despite having viable cells within the host tissue, presumably capable of a paracrine stimulus, seeded chondrocytes did not migrate into the region of cell death adjacent to the site of injury. Others have speculated that this type of cell movement may be possible[14-17] but a lack of cell migration was observed in our model despite the use of osteoarthritic cartilage which represents a more permissive environment to cell movements due to the decreased matrix density and increased "pore size". This work also highlighted the importance of cartilage tissue manipulation and the effects this can have on cell viability within the tissue. Despite the deliberate minimal use of low pressure and friction to create the AC defects a zone of cell loss adjacent to the defect still developed. This cell loss has been seen in other work[18] and it is clear that a greater understanding of the microenvironment at the host-tissue interface is essential if effective construct integration is to be achieved.

Following the development of the explant model attention was then turned to the cellular aspects of AC tissue engineering. The use of *in vitro*-expanded autologous chondrocytes is currently considered the optimal cell source for the repair of AC defects[19]. Autologous chondrocytes have been used for ACI for over a decade and despite criticisms of this technique, several studies demonstrate that it has real patient benefit. However, improvements are required to make this technique more reliable, durable, and applicable to larger defects[20,21]. One problem associated with ACI is that it utilizes chondrocytes expanded in monolayer culture resulting in loss of the differentiated chondrocyte phenotype[22-24]. In an attempt to rectify this problem and generate a group of cells with enhanced cartilage matrix-forming capacity, we first sought to further characterize chondrocytes expanded in monolayer. Consequently, the pattern of chondrocyte gene expression from *in situ* through to passage 7 focusing on the levels of collagen type I, collagen type II and aggrecan gene expression was studied. These novel results demonstrated that when chondrocytes were released from their surrounding matrix there was an immediate and rapid change in gene expression, so much so that they had lost significant chondrocyte characteristics at least by passage 0 if not sooner. By the end of passage 0, gene expression levels for collagen type I had risen ~1000x while levels for collagen type II had also risen but only by ~10x when compared to the levels obtained from chondrocytes *in situ*. In contrast, gene expression levels for aggrecan had decreased by ~10x in the same time period. These changes in gene expression levels, especially the large changes associated with collagen type I, suggested strongly that the cultured cells

can no longer be considered as chondrocytes and should be seen only as chondrocyte-like at best. This appears to be the major limiting factor in the production of a more hyaline-like repair construct. If cultured cells are to be implanted at sites of injury it would seem appropriate to suggest that the gene expression values should be returned to those found *in situ* when implanted if any tissue repair is to be eventually successful.

Much of the work attempting to restore *in situ* chondrocyte phenotype has involved passaging the cells three or more times and then modulating the cellular environment with techniques such as restoration of the rounded chondrocyte shape, medium supplementation with biomolecules, alteration in oxygen tension, or growth in high-density cultures[25-29]. Therefore, attempts were made to prevent early phenotypic alterations affecting collagen type I expression by placing chondrocytes in high density cultures immediately following digestion. In this study, it was thought that by allowing the cells to be in close contact during culture, a microenvironment conducive to chondrocyte re-differentiation would be achieved early in culture with resultant maintenance of chondrocyte genotype. This was accomplished with regards to levels of collagen type II mRNA as noted by the initial increase that was maintained as long as the culture was maintained at confluence. Conversely, in the populations of cells that were simply passaged, a smaller initial increase in collagen type II mRNA levels occurred but this quickly decreased to levels below those found *in situ*. While these results were encouraging they were countered by the upregulation of collagen type I mRNA. Immediately following release from the AC matrix, the chondrocyte populations

in both the passaged and confluence situations increased their levels of collagen type I mRNA expression and these increased levels were maintained throughout the experiment in a parallel pattern. While the results for the levels of mRNA for collagen type II were encouraging as an indicator for maintenance of normal chondrocyte genotype during confluence culture, the results related to mRNA levels of collagen type I were discouraging. Thus, collagen type I upregulation may represent the major obstacle facing chondrocyte culture in preparation for more successful transplantation at sites of AC injury. To be successful, a method must be found to either prevent this appearance of collagen type I or eliminate its appearance prior to implantation.

In an attempt to solve the deficiencies encountered when utilizing *in vitro* expanded chondrocytes, attention was then turned to MSCs as a viable alternative. MSCs are particularly attractive because they represent an autologous, easily isolated, rapidly expandable, multipotent cell source with known chondrocyte-like capacity[30]. Unfortunately, MSCs are also not without their own shortcomings and represent a heterogeneous and dynamic population of cells, comprised of a mixture of multi- and bi-potent progenitors, lineage restricted precursors, and fibroblasts[31]. To date no group has successfully identified a relationship between a MSC subpopulation and the multipotent progenitors responsible for generating cartilage. Towards this end a subpopulation of MSCs was isolated which displayed markers considered to enhance chondrogenic potential. It was thought that by generating a more uniform population of cells the potential for AC tissue repair would be enhanced. A CD44+ population of cells was isolated and found

to display a larger relative increase in both collagen type II and aggrecan gene expressions when compared to both the mixed and CD44- populations. Unfortunately, the levels of collagen type I gene expression were also increased in all three populations of cells explored when compared to the undifferentiated MSC population. As mentioned previously, the presence of collagen type I represents a significant hurdle which must be surpassed because it is not a component of normal AC. Indeed, one of the arguments against using mature chondrocytes for AC tissue engineering is that they rapidly loose their phenotype, producing increased amounts of collagen type I, leading to formation of non-durable fibrocartilage. In fact, the gene expression profiles observed by the MSC-derived chondrocytes in this study resemble closely those of dedifferentiated early passage chondrocytes observed in previous work. This again leads to questions regarding the nature and definition of a chondrocyte. Traditionally, a combination of the production of collagen type II and an absence of collagen type I has loosely defined an articular chondrocyte once it is removed from its native matrix. If this definition is utilized, the cells generated during the CD44 purification study, even those in the CD44+ population, could only be viewed as chondrocyte-like at best because the population produced both collagen type I and collagen type II.

This work highlighted many of the complications associated with repair of acute articular cartilage defects and made important advancements which will aid us as we work toward a solution to this complex problem. The development of a viable human explant model was an important first step towards this process. An appropriate *in vitro* model is essential for the early study of therapeutic

interventions so that insight can be gained prior *to in vivo* application. The explant model was successful for the purpose of this work but it was also clear that further work is required to generate a model which more closely replicates the intra-articular microenvironment, including development in the areas of nutrient delivery, oxygen tension and the application of a mechanical stimulus. Further work is also required to elucidate the nature and character of the zone of cell loss which occurs when articular matrix is damaged. The successful integration of any construct will be strongly influenced by the native matrix microenvironment adjacent to the site of injury.

The main cellular therapies being studied and applied clinically were also addressed in this work. The significant findings here revealed that there is an increase of collagen type I in both mature chondrocytes and MSCs. If we persist in utilizing cells which produce elevated levels of collagen type I to repair AC defects the only attainable result will be a hyaline-like or fibrocartilagenous repair which lacks the biomechanical durability to affect a long term cure. Further work is certainly required to further elucidate the mechanisms responsible for the unwanted genetic upregulation of the collagen type I gene and ways to decrease its expression. This will undoubtedly involve work with the cellular microenvironments including appropriate nutrient supplementation, growth factors and oxygen tension. What was most clear though is that classifying a cell as a chondrocyte based simply on the fact that it can produce collagen type II does not make it a chondrocyte. Much of the work in this field has relied on this assumption. It will only be through a more thorough chondrocyte characterization

and universal definition of what constitutes a chondrocyte that this work will eventually progress.

7.2 Reference List

1. Reginster,J.Y. The prevalence and burden of arthritis. *Rheumatology. (Oxford)* **41 Supp 1**, 3-6 (2002).

2. Elders,M.J. The increasing impact of arthritis on public health. *J. Rheumatol. Suppl* **60**, 6-8 (2000).

3. Buckwalter,J.A., Saltzman,C. & Brown,T. The impact of osteoarthritis: implications for research. *Clin. Orthop. Relat Res.* S6-15 (2004).

4. Brooks,P.M. Impact of osteoarthritis on individuals and society: how much disability? Social consequences and health economic implications. *Curr. Opin. Rheumatol.* **14**, 573-577 (2002).

5. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage.* **10**, 432-463 (2002).

6. Buckwalter,J.A. Sports, joint injury, and posttraumatic osteoarthritis. *J. Orthop. Sports Phys. Ther.* **33**, 578-588 (2003).

7. Buckwalter,J.A., Saltzman,C. & Brown,T. The impact of osteoarthritis: implications for research. *Clin. Orthop. Relat Res.* S6-15 (2004).

8. Buckwalter,J.A. Articular cartilage injuries. *Clin. Orthop. Relat Res.* 21-37 (2002).

9. Alford,J.W. & Cole,B.J. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am. J. Sports Med.* **33**, 295-306 (2005).

10. Hunziker,E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis. Cartilage.* **10**, 432-463 (2002).

11. Langer,R. & Tirrell,D.A. Designing materials for biology and medicine. *Nature* **428**, 487-492 (2004).

12. Griffith,L.G. & Naughton,G. Tissue engineering--current challenges and expanding opportunities. *Science* **295**, 1009-1014 (2002).

13. Lutolf,M.P. & Hubbell,J.A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotechnol.* **23**, 47-55 (2005).

14. Morales,T.I. Chondrocyte moves: clever strategies? *Osteoarthritis. Cartilage.* **15**, 861-871 (2007).

15. Chang,C., Lauffenburger,D.A. & Morales,T.I. Motile chondrocytes from newborn calf: migration properties and synthesis of collagen II. *Osteoarthritis. Cartilage.* **11**, 603-612 (2003).

16. Lee,G.M. & Loeser,R.F. Cell surface receptors transmit sufficient force to bend collagen fibrils. *Exp. Cell Res.* **248**, 294-305 (1999).

17. Maniwa,S. *et al.* Effects of hyaluronic acid and basic fibroblast growth factor on motility of chondrocytes and synovial cells in culture. *Acta Orthop. Scand.* **72**, 299-303 (2001).

18. Archer,C.W., Redman,S., Khan,I., Bishop,J. & Richardson,K. Enhancing tissue integration in cartilage repair procedures. *J Anat.* **209**, 481-493 (2006).

19. Peterson,L., Brittberg,M., Kiviranta,I., Akerlund,E.L. & Lindahl,A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. *Am. J. Sports Med.* **30**, 2-12 (2002).

20. Yang,K.G. *et al.* Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. *Tissue Eng* **12**, 2435-2447 (2006).

21. Hardingham,T.E., Oldershaw,R.A. & Tew,S.R. Cartilage, SOX9 and Notch signals in chondrogenesis. *J Anat.* **209**, 469-480 (2006).

22. Benya,P.D., Padilla,S.R. & Nimni,M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313-1321 (1978).

23. Benya,P.D., Padilla,S.R. & Nimni,M.E. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. *Biochemistry* **16**, 865-872 (1977).

24. Cheung,H.S., Harvey,W., Benya,P.D. & Nimni,M.E. New collagen markers of 'derepression' synthesized by rabbit articular chondrocytes in culture. *Biochem. Biophys. Res. Commun.* **68**, 1371-1378 (1976).

25. Benya,P.D. & Shaffer,J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**, 215-224 (1982).

26. Francioli,S.E. *et al.* Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: Relevance for Automated Bioreactor Systems. *Tissue Eng* (2007).

27. Schulze-Tanzil,G. *et al.* Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. *Cell Tissue Res.* **308**, 371-379 (2002).

28. Domm,C., Schunke,M., Steinhagen,J., Freitag,S. & Kurz,B. Influence of various alginate brands on the redifferentiation of dedifferentiated bovine articular chondrocytes in alginate bead culture under high and low oxygen tension. *Tissue Eng* **10**, 1796-1805 (2004).

29. Domm,C., Schunke,M., Christesen,K. & Kurz,B. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. *Osteoarthritis. Cartilage.* **10**, 13-22 (2002).

30. Baksh,D., Song,L. & Tuan,R.S. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J. Cell Mol. Med.* **8**, 301-316 (2004).

31. Phinney,D.G. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle* **6**, 2884-2889 (2007).