A METHOD TO IMPROVE CARTILAGE INTEGRATION

by

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Abstract

One major barrier that prevents cartilage integration following mosaic arthroplasty is the presence of a zone of chondrocyte death (ZCD) that is generated upon osteochondral graft harvest, which can extend up to 400 μm into the cartilaginous portion of the graft. In order for cartilage integration to occur, chondrocytes must be present at the graft periphery; however chondrocyte migration through the ZCD to the graft periphery is inhibited by the dense extracellular matrix (ECM) of cartilage. The purpose of this study was to develop a method for increasing the number of chondrocytes within the ZCD and at the periphery of a cartilage graft. This method used a combination of collagenase treatment (as a means of degrading the ECM within the ZCD) and chondrocyte chemotaxis (as a means of improving chondrocyte migration into the ZCD and to the cartilage periphery). Results indicate that treating bovine articular cartilage with 0.6 % collagenase for 10 min decreased with extent of the ZCD by approximately 35% (*collagenase:* 109 ± 13 μm; *control:* 175 ± 13 μm). Each of the chemotactic agents tested (PDGF-bb, bFGF, and IGF-I) were found to induce bovine chondrocyte chemotaxis at concentrations of 25 ng/mL in modified Boyden chamber experiments. However, in bovine articular cartilage samples that were pre-treated with collagenase (0.6% for 10 min), supplementation with 25 ng/mL of either PDGF-bb or bFGF had no apparent effect on the ZCD relative to samples treated only with collagenase (*PDGF-bb:* 85 ± 10 μm; *bFGF:* 88 ± 10 μm). Alternatively, bovine articular cartilage samples pre-treated with collagenase (0.6% for 10 min) and supplementation with 25 ng/mL IGF-I resulted in an approximately 65% reduction in the ZCD relative to samples treated only

with collagenase (*IGF-1:* 38 ± 5 μm). Thus, treating osteochondral grafts with collagenase and IGF-1 induces chondrocyte repopulation of the zone of chondrocyte death generated by osteochondral graft harvesting, and could enhance cartilage integration after implantation.

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List of Acronyms

OA	Osteoarthritis
ECM	Extracellular matrix
ZCD	Zone of chondrocyte death
MSC	Mesenchymal stem cell
IGF-I	Insulin-like growth factor I
PDGF	Platelet derived growth factor
bFGF	Basic fibroblast growth factor
PG	Proteoglycan
GAG	Glycosaminoglycan
ACI	Autologous chondrocyte implantation
FBS	Fetal bovine serum
ITS	Insulin-transferrin-selenium
BSA	Bovine serum albumin
UV	Ultraviolet
PBS	Phosphate buffered saline
HEPES	*N*-2-hydrocyethylpuperazine-*N'*-2-ethanesulfonic acid
mGC	Methacrylated glycol chitosan

Chapter 1

Introduction

1.1 Osteoarthritis in society

Osteoarthritis (OA) is one of the most common diseases in the world, affecting approximately 10% of the Canadian population (Health Canada, 2003). OA is a disease that affects the cartilage and underlying bones comprising synovial joints. Synovial joints are those joints in the body that provide contact and allow for movement between articulating bones (e.g., the knee and elbow). The ends of the articulating bones comprising synovial joints are covered with a layer of articular cartilage, which, when combined with the synovial fluid contained within synovial joints, allows joint movement to be nearly frictionless. In addition to allowing nearly frictionless movement of synovial joints, articular cartilage is also able to act as a shock-absorbing material that reduces the amount of force applied to respective articulating bones.

OA is characterized by the loss of cartilage in articulating joints, resulting in a painful condition that limits joint movement and can lead to deformity (Buckwalter, 1997) and is related to a lower quality of life (Reginster, 2002, Salaffi *et al.*, 2005). As OA worsens, the muscles and ligaments surrounding the affected joints may begin to atrophy due to decreased movement, resulting in additional pain and further restricting movement. While the underlying causes of most cases of OA are unknown, it is a disease that is generally associated with ageing, although it is known that OA can be induced by trauma and certain diseases in a small percentage of cases (Buckwalter, 1997).

In addition to the physical suffering caused by OA, it is also associated with significant costs. It has been estimated that it costs over $500 to treat a single OA patient every year (Lanes *et al.*, 1997), with OA-related costs accounting for 1-2.5% of the gross national product of Western nations (March and Bachmeier, 1997). Additionally, arthritis-related pain relates to lower productivity in the workplace, at an estimated cost of $10 billion in the United States alone (Stewart *et al.*, 2003). In Canada, it is estimated that individuals suffering from OA spend an average of $12 200 every year in order to manage their condition, not including prescription and non-prescription drugs and other therapies (Gupta *et al.*, 2005).

1.2 Articular cartilage injury

One of the main reasons why OA is such a devastating disease is the lack of healing that occurs in cartilage tissue (Mankin, 1982). Furthermore, injuries to cartilage will worsen progressively over time, causing even minimal cartilage defects to eventually result in a state of OA (Messner and Gillquist, 1996). While there are currently several treatment options available for those suffering from OA, none result in the reformation of high quality articular cartilage within synovial joints (Hunziker, 2002).

Injuries that affect articular cartilage can be classified into one of two categories: 1) chondral injuries, and 2) osteochondral injuries. Chondral injuries involve only the articular cartilage and do not result in any spontaneous healing response. One of the main reasons why chondral injuries do not undergo spontaneous regeneration is that cartilage has a densely-woven extracellular matrix (ECM); the chondrocytes (cartilage cells) are

physically unable to migrate through the ECM to the injury site to initiate a healing response (Qiu *et al.*, 2000). Additionally, because cartilage is an avascular tissue (*i.e.*, it has no blood supply), cartilage injuries do not result in the rupture of blood vessels and subsequent release of marrow-derived stem cells into the defect region that are able to initiate a healing response (Messner and Gillquist, 1996). Osteochondral injuries involve not only the articular cartilage, but also the underlying subchondral bone. Such injuries result in spontaneous repair as the subchondral bone contains blood vessels that rupture and result in a clot formation, which serves as a basis for spontaneous wound healing (Hunziker, 2002). Subchondral bone bleeding will release mesenchymal stem cells (MSCs) into the defect region (Beris *et al.*, 2005), and will result in repair tissue (fibrocartilage) filling the defect region (Messner and Gillquist, 1996). However, the fibrocartilage that fills the defect region has inferior mechanical properties relative to normal articular cartilage, and wears away over time, leaving exposed subchondral bone (Messner and Gillquist, 1996; Giffin *et al.*, 2001; Hunziker, 2002).

1.3 Surgical repair of articular cartilage

Several surgical strategies have been developed in an attempt to mitigate the pain and suffering caused by articular cartilage defects and OA. One of the earliest options for patients suffering from joint pain caused by OA was total joint replacement. However, joint replacement is not generally recommended for patients under the age of 55 due to high rates of failure of the prosthetic joints (Richmond, 2009). Failure rates at 10 years of prosthetic joints can be two and a half times higher in patients under the age of 55 relative

to patients over the age of 70 (Rand *et al.*, 2003). Additionally, prosthetic joints can suffer from loosening and can have poor wear characteristics (Nesic *et al.*, 2006). In an attempt to avoid the problems associated with total joint replacements, researchers developed procedures to delay the need for a prosthetic joint by using the body's own intrinsic healing ability. The early goal of surgeons was to alleviate pain in the affected joint. This led to strategies where the surgeon would attempt to improve the congruity of the affected joint surface by shaving away pieces of malformed and torn cartilage. Such procedures are known as debridement. These procedures have been shown to bring about a reduction in pain at 1 year, but results indicate that there is significant deterioration in patients at 5 years (Shah *et al.*, 2007).

Another strategy employed for treating cartilage defects is the microfracture technique. The purpose of microfracture is to create a more congruous surface by filling the defect region with repair tissue. In general, defect filling is accomplished by puncturing the underlying subchondral bone and allowing blood, along with MSCs, to fill the defect region. The MSCs will then generate fibrous tissue that fills the defect (Furukawa *et al.*, 1980). However, the fibrous tissue is not like normal hyaline cartilage (cartilage that is specific to synovial joints), and wears away over time (Nehrer *et al.*, 1999). Thus, such strategies are associated with improved joint function and decreased pain (Scopp and Mandelbaum, 2005), but results begin to deteriorate after 18-24 months (Kessler *et al.*, 2008).

Mosaic arthroplasty, also know as osteochondral grafting or transfer, is a cartilage repair technique that aims to restore the articulating surface in a joint where a chondral or

osteochondral defect has occurred. Mosaic arthroplasty involves the transfer of healthy osteochondral tissue into a defect region. The osteochondral grafts are typically taken from relatively non-weight bearing regions of the affected joint and implanted into defect regions that have been prepared for graft insertion (Figure 1.1). Alternatively, if the defect to be repaired is large (> 7 cm^2), allograft tissue from a donor may be used (Aubin *et al.*, 2001; Gross, 2002). One of the main benefits to mosaic arthroplasty is that it immediately results in filling of the defect region with high quality hyaline cartilage. Therefore, patients who undergo mosaic arthroplasty have faster recovery times than patients receiving other treatments, and are often able to participate in physical activities sooner after undergoing the procedure (Horas *et al.*, 2003).

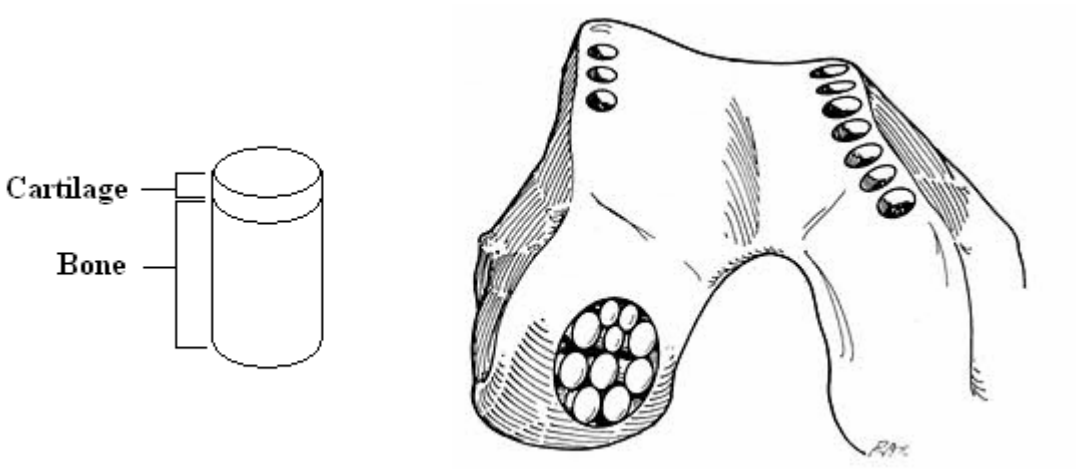

Figure 1.1. Osteochondral grafts from non-weight-bearing regions of the knee are transplanted into a defect region during mosaic arthroplasty (adapted from www.southalabama.edu/usahealthsystem/jointrestoration/cartilage.html).

While mosaic arthroplasty has had success as a treatment for OA and other cartilage diseases, there are several problems associated with it that may have detrimental effects on clinical outcomes. One of the main problems with mosaic arthroplasty is the lack of integration between the cartilaginous portions of the grafts and the surrounding cartilage tissue (Huntley *et al.*, 2005). One of the main reasons for such a lack of integration is that the chondrocytes are unable to migrate through the dense ECM that comprises the structural components of cartilage (Qiu *et al.*, 2000). Therefore, the chondrocytes are unable to reach the edges of the grafts and generate new cartilage that would allow the grafts and surrounding cartilage to integrate. However, if such problems can be overcome, clinical outcomes of patients undergoing mosaic arthroplasty may be increased.

1.4 Regenerative medicine approaches for cartilage repair

Surgical repair options for cartilage defects offer limited healing potential and could be improved upon. One field of research that aims to improve cartilage healing is tissue engineering. The objective of tissue engineering is to provide repair tissue in an off-the-shelf manner without need for donor allograft tissue. For example, one aim of tissue engineering is to someday generate hearts for patients requiring heart transplants without requiring a donor heart. The tissue engineering approach combines a scaffold with cells from the patient's own body in order to grow new tissue *in vitro* in a laboratory that can be implanted into the patient. Another similar strategy for cartilage repair is the regenerative medicine approach, which aims to generate new tissue either via tissue

engineering, or with cell and/or drug therapy. While not completely separate from classical tissue engineering, the regenerative medicine approach could allow for tissue formation *in situ*, rather than *in vitro*. For example, a regenerative medicine approach could involve the implantation of a scaffold into a defect region and recruit into it cells from surrounding tissue that are able to generate new tissue *in situ* without the need for growing new tissue in a laboratory. Therefore, the regenerative medicine approach is a one-step procedure, unlike tissue engineering, which requires two steps – a cell harvest step and tissue implantation step following tissue culture.

The regenerative medicine approach to cartilage repair has not been explored in depth, perhaps due to the difficulty in recruiting differentiated chondrocytes into a cartilage defect. The current regenerative medicine approaches for cartilage repair have involved recruiting MSCs from the underlying subchondral bone. These approaches have had some success in regenerating cartilage that is able to fill the defect, but the resulting tissue contains significant amounts of fibrous tissue and fibrocartilage (Erggelet *et al.*, 2007).

1.5 Research outline

The goal of this research is develop a method to improve cartilage integration in osteochondral transfer procedures. Previous methods that have been investigated to improve cartilage integration have included treatment of cartilage with ECM-degrading enzymes. However, these studies did not look systematically at how to treat cartilage to improve integration, and did not consider clinically-viable treatment times. The goal of

the present research is to develop a treatment method wherein a surgeon can treat an osteochondral graft with ECM-degrading enzymes for a short period of time so that cartilage defects can be treated in a single surgical procedure. Additionally, the present research will investigate the use of a chemotactic agent as a method to increase chondrocyte migration through the ECM to the graft edge to promote cartilage integration. Finally, this research will investigate the suitability of using a novel photocrosslinkable hydrogel, methacrylated glycol chitosan, in order to fill the void regions between grafts following osteochondral transfer. The results of this research may indicate better ways to perform mosaic arthroplasty, and may help promote cartilage integration in other procedures where cartilage integration can be improved (*i.e.* autologous chondrocyte implantation, cell/scaffold transplants).

Chapter 2

Literature Review

2.1 Articular cartilage structure and function

Articular cartilage is a tissue found on the ends of articulating joints (i.e. knees, hips, elbows) that allows for smooth, gliding movement between two bones in the body. Articular cartilage is composed of chondrocytes (cartilage cells) distributed within ECM. The chondrocytes are the only cells present within articular cartilage. The ECM is composed mainly of type II collagen and proteoglycans and can be organized into four distinct zones. Because of its composition and structure, articular cartilage is a resilient tissue that is able to withstand the various forces imparted on articulating joints.

2.1.1 Chondrocytes

Chondrocytes are the resident cells in cartilage and comprise approximately 1.5-2.0 % of the volume of cartilage tissues (Hunziker, 2002). While chondrocytes occupy such a relatively small volume in the tissue, they are solely responsible for ECM maintenance. Chondrocytes are derived from the mesenchymal lineage, with differentiation occurring during skeletal development (Kuo *et al.*, 2006). However, unlike other cells that are derived from the mesenchymal lineage (i.e., bone cells), chondrocytes are unable to initiate a healing response as a result of injury of the tissue (Caplan *et al.*, 1997).

Cartilage is an avascular tissue and chondrocytes must therefore derive nutrients from the synovial fluid contained within the joint. However, as synovial fluid has low oxygen and nutrient availability, chondrocytes are unable to generate significant amounts of energy through aerobic respiration. Therefore, chondrocytes rely mostly on anaerobic respiration to generate ATP, and are accordingly less metabolically active than other cells found in the body (Lane *et al.*, 1977). The level of chondrocyte activity is also dependent on the location of the chondrocytes within the tissue. Chondrocytes located in the deep and calcified zones of articular cartilage have the highest level of mitotic activity and have the greatest potential to produce new tissue (Archer *et al.*, 1990, Bos *et al.*, 2008).

2.1.2 Chondrocyte migration and chemotaxis

Until recently, chondrocytes have been thought to be incapable of migration, and this has been mentioned by some researchers as a potential reason for lack of healing in cartilage tissues (Kinner *et al.*, 2005; Khan *et al.*, 2008). When other tissues in the body are injured, a healing cascade is initiated whereby there is significant cellular migration to the injury site. However, it has only recently been shown that chondrocytes are capable of migration in response to certain agents in a process known as chemotaxis (Morales, 2007; Hidaka *et al.*, 2006). Chondrocytes have been shown to migrate in response to several chemotactic agents, including insulin-like growth factor I (IGF-I) (Chang *et al.*, 2003), platelet derived growth factor (PDGF) (Fujita *et al.*, 2004; Fukuyama *et al.*, 2004; Mishima and Lotz, 2008), basic fibroblast growth factor (bFGF) (Hidaka *et al.*, 2006; Maniwa *et al.*, 2001), and other agents (Morales, 2007). While chondrocyte migration

was first shown to occur on planar surfaces in two-dimensional environments, it has been demonstrated that chondrocytes are capable of polarizing in the direction of the chemoattractant stimulus while within cartilage as well (Morales, 2007).

One of the main barriers to chondrocyte migration *in situ* is the dense ECM that chondrocytes have to migrate through in order to reach the wound edge (Hunziker *et al.*, 1998). It has been demonstrated that when ECM components are broken up by use of ECM-degrading enzymes, chondrocytes are able to migrate to the edge of wounded cartilage (Qiu *et al.*, 2000). Several enzymatic solutions have been used to degrade the ECM in cartilage in order to improve chondrocyte migration through the ECM, including chondroitinase ABC (Hunziker *et al.*, 1998; Qiu *et al.*, 2000), trypsin (Qiu *et al.*, 2000), collagenase (Qiu *et al.*, 2000; Bos *et al.*, 2002), and hyaluronidase (Qiu *et al.*, 2000, Bos *et al.*, 2002, Janssen *et al.*, 2006), and various combinations of these enzymes (Hunziker *et al.*, 1998; Quinn *et al.*, 2002; van de Breevaart Bravenboer *et al.*, 2004; Janssen *et al.*, 2006). However, the ECM-degrading enzyme treatment times used in these studies have generally not been of clinical significance, with some treatment times being as long as 48 hours. Nevertheless, one research group (Janssen *et al.*, 2006), recognized the need to decrease the treatment time so that it could be used by a surgeon in a single-step operative procedure. The use of ECM-degrading enzymes has been shown to not only allow for improved chondrocyte migration through the dense ECM, but also allows for improved integration between cartilage explants *in vitro* and *in vivo* (Janssen *et al.*, 2006).

Chondrocyte migration occurs via a five-step process (Morales, 2007): 1) polarization of the cell body in the direction of the stimulus (*i.e*. concentration gradient), 2) protrusion formation, 3) regulation of adhesion to the substratum, 4) contraction of the cell body, and 5) detachment of the rear of the cell body. It has also been suggested that chondrocytes migrate along the length of collagen fibers in order to migrate in three-dimensional environments (Morales, 2007).

2.1.3 Extracellular matrix components

The ECM of articular cartilage has a specific composition, which is unlike that of other cartilage. It is mainly composed of collagen and proteoglycans, with trace amounts of other proteins and lipids. The ECM in articular cartilage is able to withstand the various forces applied to the joint and contains protein on the surface known as lubricin, which allows for nearly frictionless movement within a joint. The basic components of articular cartilage are illustrated in Figure 2.1.

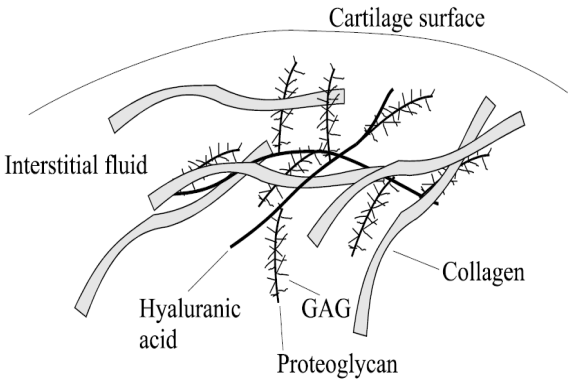

Figure 2.1. Extracellular matrix composition of articular cartilage (adapted from http://engineering.union.edu/~rapoffa/MER445/Module%206%20Articular%20Cartilage/Function%20&%20Structure%20of%20Articular%20Cartilage.pdf).

2.1.3.1 Collagens

Collagens are the most predominant macromolecules in articular cartilage, accounting for 10-30% of the wet weight of the tissue (Cohen *et al.*, 1998). The main type of collagen in articular cartilage is type II collagen, which accounts for 90-95% of the total amount of collagen within articular cartilage (Buckwalter, 1997). Articular cartilage also contains smaller amounts of types V, VI, IX, X, and XI collagen (Buckwalter, 1997).

Collagen fibrils are polypeptides that are composed mainly of glycine, proline, and hydroxyproline. The general amino acid sequence in a collagen fibril is glycine-X-Y, where X is any amino acid, and Y is proline or hydroxyproline. A single fibril contains three polypeptide chains organized into a triple-helical structure. Multiple fibrils come together to form collagen fibers, which imparts cartilage with high tensile strength and

shear resistance. The thickness of individual collagen fibers varies depending on the location within articular cartilage (Cohen *et al.*, 1998; Buckwalter, 1997).

2.1.3.2 Proteoglycans

Proteoglycans (PGs) account for 3-10% of the wet weight of articular cartilage and allow the tissue to distribute load from an impact over a wide area, decreasing the force applied to a specific point within a joint. PGs are complexes that contain a protein core with branched side-chains known as glycosaminoglycans (GAGs). The most common PG in articular cartilage is aggrecan, which accounts for 80-90% of all PGs in the tissue. Multiple PGs will bind to a hyaluronan macromolecule, creating a PG aggregate that interacts with collagen to aid in collagen retention within the tissue (Cohen *et al.*, 1998).

The GAG chains contain significant amounts of carboxyl and sulphate groups, most commonly chondroitin sulphate and keratin sulphate, which become negatively charged when dissolved in interstitial fluid and are thus hydrophilic. The hydrophilic nature of GAG chains causes articular cartilage to be highly hydrated, and generate a swelling pressure within the cartilage (Cohen *et al.*, 1998), which allows the tissue to resist compressive deformation.

2.1.3.3 Water

Water accounts for 65-80% of the wet weight of articular cartilage (Buckwalter, 1997) and provides a medium for nutrient diffusion throughout the tissue to the chondrocytes. Water is also an important component in articular cartilage from a

mechanical perspective, allowing for lubrication between joint surfaces and being primarily responsible for the tissue's compressive-bearing ability (Buckwalter, 1997).

2.1.4 Zonal organization

Articular cartilage contains four distinct zones, which cover the subchondral bone, each of which has a different biochemical composition, water content, and cellular orientation (Figure 2.2). The four zones are: 1) the superficial tangential zone, 2) the middle or transition zone, 3) the deep zone, and 4) the calcified zone. The superficial tangential zone is the outermost region and accounts for 10-20% of the total thickness of articular cartilage (Buckwalter, 1997). Within this region, collagen fibrils are thin and oriented parallel to the articulating surface and chondrocytes have a flattened morphology. The superficial tangential zone contains the highest proportion of collagen and water relative to the other zones, and the lowest proportion of proteoglycans. The outermost layer of the superficial tangential contains significant amounts of lubricin (also known as superficial zone protein), which along with synovial fluid, allow for nearly frictionless movement between two joints (Ohno *et al.*, 2006). The middle or transition zone lies under the superficial tangential and accounts for 40-60% of the total thickness of articular cartilage. Within this region, collagen fibrils are randomly oriented and the chondrocytes have a rounded morphology. The middle zone contains moderate amounts of collagen and proteoglycans, and has water content that is less than the superficial tangential zone. The deep zone lies under the middle zone and accounts for approximately 30% of the total thickness of articular cartilage. Within this region, the

collagen fibrils are oriented perpendicular to the articulating surface and the chondrocytes have a spherical morphology and a columnar organization. Water content within this zone is lower than in the middle zone, and the PG content is the highest (Buckwalter, 1997). The calcified zone is a hard transition zone between hyaline cartilage (all articular cartilage above the calcified zone) and the underlying subchondral bone.

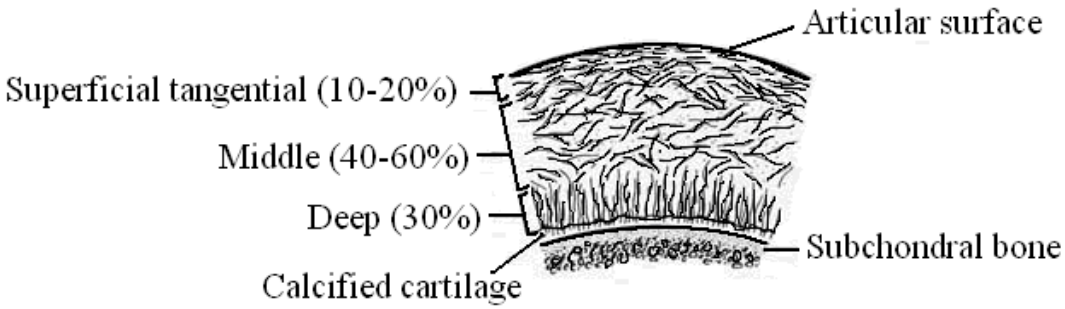

Figure 2.2. Articular cartilage is organized into distinct zones, where the physical and chemical structure between each zone differs (adapted from http://www.engin.umich.edu/class/bme456/cartilage/cart.htm)

2.2 Surgical repair technologies for articular cartilage

While no technology currently exists that is able to induce consistent regeneration of high quality, hyaline-like articular cartilage, there are several technologies available that can provide patients suffering from cartilage injuries and diseases with decreased pain and increased mobility. The available technologies can be classified into two categories (Buckwalter, 1997): 1) injury palliation techniques, and 2) resurfacing techniques.

2.2.1 Defect classification

Articular cartilage defects are classified based on whether they penetrate through the cartilage to the subchondral bone. Thus, defects are classified into one of two categories: 1) chondral defects that affect the articulating surface of the cartilage, and 2) osteochondral defects that affect the articulating surface and the underlying subchondral bone. Chondral defects do not result in any sort of spontaneous repair (Buckwalter and Mankin, 1998), and will worsen over time, eventually resulting in an osteoarthritic state (Kleeman *et al.*, 2005). Osteochondral defects result in spontaneous repair, as breaching of the subchondral bone results in bleeding and an influx of MSCs into the defect region (Messner and Gillquist, 1996; Beris *et al.*, 2005). As blood fills the defect region, a clot is formed, and the mesenchymal stem cells are able to generate scar tissue that fills the defect region (Furukawa, 1980; Shapiro *et al.*, 1993). This scar tissue is fibrous tissue or fibrocartilage, which has inferior properties relative to hyaline cartilage in terms of mechanical properties and durability (Hunziker, 2002). This tissue does not integrate with the surrounding hyaline cartilage and begins to wear away after 12 weeks (Shapiro *et al.*, 1993).

2.2.2 Injury palliation techniques

Historically, surgical interventions for the treatment of chondral and osteochondral defects have been generally aimed at symptom relief rather than regeneration of articular cartilage. Such palliative techniques aim to make the articulating surface smoother, allowing for better movement of the affected joint.

2.2.2.1 Debridement

Debridement is an arthroscopic technique that is used to treat chondral and osteochondral defects that aims to smooth out the articulating surface by removing surface imperfections in articular cartilage. The use of debridement as an effective treatment method for those suffering from OA is questioned by many researchers (Rowland, 1993, Dervin *et al.*, 2003), with some believing that any improvements in symptoms by those receiving the treatment are due to a placebo effect (Moseley *et al.*, 2002). Indeed, it has been mentioned that there is no biological rationale for performing debridement, and the procedure may instead lead to decreased joint mobility (Hunziker, 2002). However, there are proponents of debridement who believe that it is a worthwhile palliative procedure when performed on patients who will have a predictable outcome from undergoing the procedure (Jackson and Dieterichs, 2003). Results from debridement studies vary, with some studies reporting good clinical outcomes (Baumgaertner *et al.*, 1990) and other studies reporting poor clinical outcomes (McGinley *et al.*, 1999). However, even in patients who have decreased symptoms following debridement, the benefits of undergoing such a procedure significantly deteriorate by 5 years (Shah *et al.*, 2007). One thing that is agreed upon by opponents and proponents of debridement is that the procedure results in unpredictable healing outcomes, and because of this, should be more limited in its use (Fond, 2002).

2.2.2.2 Marrow stimulation techniques

Marrow stimulation techniques, such as Pridie drilling, abrasion arthroplasty and microfracture, are techniques that cause bleeding in the defect region following breaching of the subchondral bone (Sgaglione *et al.*, 2002). As the blood in the defect region clots, the mesenchymal stem cells within the clot generate fibrocartilage (Furukawa, 1980; Shapiro *et al.*, 1993) and the affected joint is subjected to controlled loading as part of post-operative care in order to promote tissue development (Sgaglione *et al.*, 2002). However, the response of the repair tissue to controlled loading and the durox ability of the fibrous tissue are unpredictable (Sgaglione *et al.*, 2002), and the repair tissue degrades over time (Scopp and Mandelbaum, 2005), with clinical results declining after 5 years (Hunziker, 2002). While it is known that fibrocartilage has properties that are inferior to articular cartilage, proponents of marrow stimulation techniques believe that filling the defect region with an inferior material is still better than leaving the defect region unfilled. Of the marrow stimulation techniques mentioned, microfracture is considered the best technique currently available, as the other techniques lead to thermal necrosis and significant removal of subchondral bone (Shah *et al.*, 2007; Steadman *et al.*, 1997; Smith *et al.*, 2005).

2.2.3 Cartilage resurfacing techniques

Cartilage resurfacing techniques aim to fill defect regions with durable hyaline cartilage. The two most widely used cartilage resurfacing techniques are autologous chondrocyte implantation and mosaic arthroplasty.

2.2.3.1 Autologous chondrocyte implantation

Autologous chondrocyte implantation (ACI) involves obtaining a cartilage biopsy from a patient, isolating the chondrocytes, expanding the number of chondrocyte *in vitro* under monolayer culture conditions, and injecting the cultured chondrocytes into the defect region under a periosteal flap (Minas, 2001). The rationale for performing ACI is that the chondrocytes injected under the perisoteal flap will, under controlled loading conditions, generate ECM that will fill the defect region. Early clinical outcomes indicated that patients who underwent ACI reported less pain and increased joint mobility following the procedure, and that the repair tissue generated was hyaline-like in nature (Brittberg *et al.*, 1994). After 2 years, one study found that 82% of patients had good to excellent results, and after an average of 7.8 years, 84% had good to excellent results (Peterson *et al.*, 2002).

Despite positive results from ACI reported by initial investigators, other researchers have found that ACI results in the formation of fibrocartilage (Hunziker, 2002) in the defect region rather than hyaline cartilage, and that there was no difference between ACI-treated defects and control defects (Breinan *et al.*, 1997). Additionally, as the chondrocytes used in ACI are expanded under monolayer conditions, they tend to dedifferentiate and produce low amounts of poor quality cartilage-like tissue (Breinan *et al.*, 1997; Hunziker, 2002; Mobasheri *et al.*, 2009). An additional study found that the repair tissue becomes more fibrous over time (Breinan *et al.*, 2001). Furthermore, it has been noted that the healing that results from ACI may not be due to the presence of chondrocytes under the periosteal flap. It has been suggested that chondrocyte leakage

from underneath the flap may be significant, and that the periosteal flap may be lost if the patient is not completely immobilized (Hunzkier, 2002). Furthermore, the periosteal flap itself can fill up to 85% of the defect region (Hunziker, 2002), and may be the most important factor modulating the repair response.

One of the main drawbacks to the ACI procedure is that it is a two-step procedure; the first step is required for cartilage biopsy, and the second step is required for chondrocyte injection into the defect region. Additionally, it is an expensive procedure, and is currently not funded by the Canadian health care system.

2.2.3.2 Osteochondral transfer (mosaic arthroplasty)

Mosaic arthroplasty, also known as osteochondral grafting or transfer, is another treatment option whereby autologous osteochondral grafts are implanted into defect regions (See Figure 1.1). Mosaic arthroplasty was originally proposed as a treatment for cartilage defects in the early 1900s (Hunziker, 2002), but was not developed as a surgical technique until 1991 (Hangody and Füles, 2003). Recovery times are on the order of approximately four to five weeks, comprising two non-weight-bearing weeks followed by two or three partial-weight-bearing weeks (Hangody and Füles, 2003). Additional benefits of mosaic arthroplasty include biocompatibility of the transplanted grafts and no risk of disease transferal between different donor sources, as may be the case when using allograft tissue (Harman *et al.*, 2006).

Several studies have looked into the clinical effectiveness of using autologous osteochondral grafts to repair cartilage defects, with encouraging short-term results (Lane

et al., 2004; Harman *et al.*, 2006). One longer-term study found that 91% of patients had good-to-excellent clinical outcomes between 3 and 6 years after mosaic arthroplasty (Hangody *et al.*, 1998). In relation to ACI, mosaic arthroplasty has been shown to result in faster recovery times (Horas *et al.*, 2003). Another study found that mosaic arthroplasty is superior to Pridie drilling, microfracture and abrasion arthroplasty in terms of clinical outcomes, especially after 3-5 years (Hangody *et al.*, 1998). Additionally, transplanted grafts are able to maintain their hyaline cartilage over time (Hangody and Füles, 2003).

Mosaic arthroplasty is recommended for repairing small and medium-sized defects (1-4 cm^2) (Hangody and Füles, 2003). This is because larger defects require too much donor tissue, which results in increased donor site morbidity (Hangody *et al.*, 1998). Larger defects of up to 8-9 cm^2 have been treated using mosaic arthroplasty (Hangody *et al.*, 1998); however, treating such large defects is usually advised against.

2.2.4 Limitations of mosaic arthroplasty

While mosaic arthroplasty results in favorable clinical outcomes, there are still several problems associated with the procedure. If these problems can be overcome, then clinical outcomes could likely be further improved.

2.2.4.1 Donor site morbidity

One of the main issues with mosaic arthroplasty is donor site morbidity. Indeed, it has been argued that mosaic arthroplasty is simply filling one defect region while creating another, and thus will fail as a surgical intervention in the long term (Hunziker, 2002).

However, research suggests that donor sites do not result in further degenerative changes of the joint (Simonian *et al.*, 1998). While the donor site has been found to fill with fibrous tissue (Iwasaki *et al.*, 2007), it is rare that patients are negatively affected by donor-site-related problems. This is likely due to the fact that the donor sites are in lesser weight-bearing regions in the knee (Hangody and Füles, 2003; Iwasaki *et al.*, 2007). Nonetheless, donor site morbidity is a potential site for development of other osteochondral defects in the joint over time, and longer-term studies are required in order to determine what effect the donor sites might eventually have.

2.2.4.2 Graft viability and the zone of chondrocyte death

Graft viability has also been questioned following implantation due to the harsh conditions they are subjected to during the mosaic arthroplasty procedure (*i.e*. hammering the graft into the defect site) (Hunziker, 2002). While it has been demonstrated that there is good survival of the cartilaginous portion of the graft, it has been found that graft harvesting results in a zone of chondrocyte death (ZCD) around the periphery of the tissue, which can extend up to 400 μm into the tissue (Figure 2.3) (Huntley *et al.*, 2005). This would mean that a typical 4.5 mm diameter graft would lose approximately 30% of its chondrocytes, all of which would be adjacent to the wound graft edge. However, depending on the instrument used, the zone can be reduced, but is still significant (Evans *et al.*, 2004; Huntley *et al.*, 2005). Additionally, when subjected to compressive forces up to 20 MPa (i.e. hammering) to implant the graft, up to 50% of chondrocytes undergo apoptosis in bovine articular cartilage explants (Loening *et al.*, 2000).

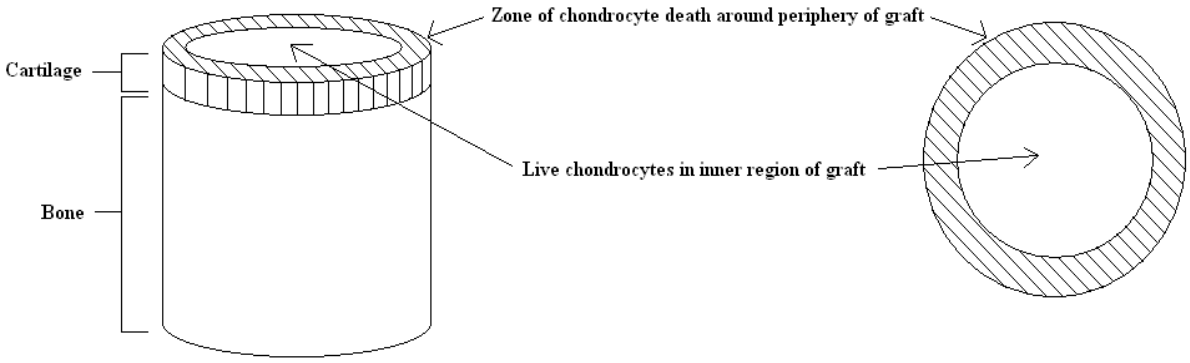

Figure 2.3. Zone of chondrocyte death in the cartilaginous portion of an osteochondral graft when viewed top-down from the cartilage surface of the graft.

2.2.4.3 Regions between grafts

A spontaneous healing response is initiated upon debridement of the defect surface during mosaic arthroplasty, resulting in fibrocartilage formation between the grafts (Mandelbaum *et al.*, 1998). The formation of fibrous tissue, in conjunction with low chondrocyte cellularity at the edges of the graft, may prevent the graft tissue from integrating with the surrounding tissue. In one study, it was noted that the bony part of the graft was well integrated after 3 months postoperatively, whereas the cartilaginous portion of the graft showed no signs of integration after 6 months (Siebert *et al.*, 2001). Additionally, it has been noted that the fibrous tissue formed between the grafts is susceptible to fissuring in large defects, which can potentially result in poor healing outcomes (Wang, 2001).

2.2.4.4 Cartilage integration

One implication regarding the zone of death associated with graft harvest is that it likely hinders graft integration with surrounding tissue. Unlike many other tissues in the body, cartilage wounds do not result in a zone of high cellularity adjacent to a wound site, which hinders the healing response (Tew *et al.*, 2000; and Sharma and Elisseeff, 2004). Thus, the presence of a zone of death would further reduce the cellularity at the wound edge.

2.3 Tissue engineering and regenerative medicine

Surgical repair options for cartilage defects offer limited healing potential and could be improved upon. Knowledge gained through tissue engineering and regenerative medicine research could someday provide better and more reliable outcomes for those suffering from cartilage injuries and OA. The tissue engineering approach aims to provide repair tissue in an off-the-shelf manner without need for donor tissue. This approach looks to generate novel tissue *in vitro* as required. The regenerative medicine approach builds upon knowledge gained through tissue engineering and aims to regenerate novel tissue *in situ*, without the need of *in vitro* tissue growth. While these approaches have distinct differences from one another, they both aim to generate high quality repair tissue that can replace damaged or worn out tissue. In many cases, both methods will use cells and scaffold in order to generate new tissue. In the tissue engineering approach, cells may be harvested from the patient requiring tissue

replacement; in the regenerative medicine approach, cells from within the body may be used to migrate to the defect region and generate new tissue.

One repair option that has been suggested for cartilage regeneration is using the body as an '*in vivo* bioreactor' by inducing cartilage formation following deliberate injury of the periosteum (Emans *et al.*, 2005). Following injury of the periosteum, hyaline cartilage has been shown to fill the defect at 10 and 20 days, but is more bone-like by 40 days (Emans *et al.*, 2005). This cartilage would then be available for transplant into a cartilage defect. One attractive feature of this repair option is that it does not require scaffold material or additional cells, although the procedure would require two surgical interventions.

It has been argued that the size of the defect being repaired will dictate the approach that is acceptable for use (Martin *et al.*, 2007). Smaller defects may be able to use a truly regenerative medicine approach, using only a cell-free scaffold. Slightly larger defects will require drug delivery to promote cell migration into the scaffold. Larger defects may require cells pre-loaded into the scaffold.

2.3.1 Cell-free scaffolds for tissue regeneration

The cell-free scaffold approach to tissue repair lies at the heart of regenerative medicine. In order to successfully regenerate tissue *in situ* within the scaffold, cells must be able to migrate from nearby tissue into the scaffold. One of the main concerns for cartilage tissue regeneration is the inability of chondrocytes to migrate through cartilage. However, as mentioned earlier, one strategy that has been developed to promote

chondrocyte migration through cartilage is by enzymatically digesting the cartilage, allowing for freer movement of chondrocytes through the tissue. However, the use of enzymatic treatment of cartilage coupled with migration into a scaffold has yet to be studied.

One strategy that has been used to repopulate a scaffold with chondrocytes *in situ* is to load the scaffold with minced cartilage that has been harvested from another area of the affected joint (Lu *et al.*, 2006; McCormick *et al.*, 2008). As the cartilage is minced, the surface area of the tissue is increased, allowing for greater chondrocyte migration out of the cartilage and into the scaffold. The benefits of such a strategy are that it requires only a single operative procedure and that the cells populating the scaffold are primary chondrocytes that have not undergone dedifferentiation as do chondrocytes that undergo *in vitro* expansion in tissue engineering approaches. Results of the minced cartilage approach have demonstrated that cells from the minced cartilage are able to migrate out of the tissue and into a scaffold, and that the migrated chondrocytes are able to generate a hyaline repair tissue in a defect region.

An early cell-free scaffold approach used a fibrin clot to fill a cartilage defect. In this study, fibrin clots were loaded with 25 μg IGF-1 and polymerized *in situ* within in a cartilage defect, with the aim of recruiting MSCs into the clot (Nixon *et al.*, 1999). The migrated MSCs generated type II collagen within the defect region, but the resulting cartilage was not hyaline cartilage, but rather fibrocartilage.

A cell-free scaffold approach without the need for drug delivery may be sufficient to repair small defects (Martin *et al.*, 2007). For larger defects, a cell-free scaffold may

require drug delivery to promote sufficient cell migration into the scaffold. Nonetheless, a cell-free scaffold method that has been studied is the use of a scaffold material implanted within a defect region that has undergone microfracture (Erggelet *et al.*, 2007). This strategy aims to provide the blood and cells that enter the defect region with a scaffold that promotes enhanced tissue formation relative to a scaffold-free approach.

2.3.2 Scaffolds for tissue repair

Selection of an appropriate scaffold material is of crucial importance, and many different materials have been investigated for potential use in cartilage tissue engineering. In general, scaffold materials can be classified into one of two groups: i) natural scaffold materials, and ii) synthetic scaffold materials. Natural scaffold materials have generated interest because they are thought to elicit little or no biological response (Darling and Athanasiou, 2004). However, concerns have been raised regarding their poor processing characteristics, potential for inducing an immune response, and lack of availability (Cheung *et al.*, 2007). Synthetic scaffold materials are easily processed, available in large supply, and can be highly modified to elicit a predictable response (Cheung *et al.,* 2007). Additionally, synthetic scaffold materials can be highly tailored in terms of their shape, chemical composition, and degradation rate (Nesic *et al.*, 2006).

There are several characteristics that a scaffold must possess in order to be considered for potential use in tissue engineering. The first consideration is biocompatibility (i.e. low immunogenic and cytotoxic response) (Hunziker, 2002). Not only should the bulk scaffold material be biocompatible, but its degradation products

should also be biocompatible (Frenkel and Di Cesare, 2004). Due to the fact that articular cartilage is an avascular and alymphatic tissue, most immune responses are relatively low compared to other tissues in the body (Darling and Athanasiou, 2004). However, a more biocompatible material is always preferred over one that elicits an immune response, especially for gaining regulatory approval.

In addition to biocompatibility, a scaffold must be biodegradable (Hunziker, 2002). The rate of degradation is a very important selection factor, as ideally, biodegradation rate would equal that of tissue formation rate (Drury and Mooney, 2003). If a scaffold degrades too quickly, the structural integrity of the scaffold would be compromised, and the underdeveloped neo-tissue alone would not be able to withstand the mechanical forces exerted on the joint (Chung and Burdick, 2008). If a scaffold degrades too slowly, it could inhibit neo-tissue formation (Chung and Burdick, 2008). It has also been shown that a slow degradation rate can result in cracks in the surface of the neo-tissue (Solchaga *et al.*, 2005).

The mechanical characteristics of a scaffold can be more or less important, depending on whether tissue formation is to occur *in vitro* or *in vivo*. If *in vitro* tissue formation is desired, then the scaffold must be able to withstand the forces it is subjected to during cultivation, such as a hydrodynamic culture vessel, and hence a mechanically strong scaffold material is not required. However, if the tissue is to be mechanically stimulated *in vitro* (as a means of increasing matrix production) (Kuo *et al.*, 2006) or grown in a dynamic loading bioreactor, then it must be able to withstand the applied forces, which are relatively small compared to the forces exerted on native cartilage

tissue *in vivo,* but are significant nonetheless. If *in vivo* tissue formation is desired, then the scaffold must be able to withstand the high compression and shear forces exerted on the joint, significantly greater than any such force encountered *in vitro*. As such, the mechanical integrity of the scaffold is crucially important, as it could otherwise fail after implantation.

Porosity is another important scaffold design criteria that should be considered. A scaffold should be porous in order to allow for cell seeding and cellular interactions (Hunziker, 2002). Porosity also allows for nutrient diffusion through the scaffold. Additionally, porosity will affect the mechanical properties of the scaffold.

A final scaffold design criteria that must be considered is how target cells interact with the scaffold. Adhesion of target cells to the scaffold is important because cells must remain on or within a scaffold in order to generate tissue within it (Yang *et al.*, 2001). Additionally, if cells are to be recruited from surrounding tissue rather than loaded into the scaffold before implantation, the cells must be able to migrate into the scaffold.

There are three basic types of scaffolds currently being investigated: i) mesh scaffolds, ii) sponge scaffolds, and iii) hydrogel scaffolds.

2.3.2.1 Mesh scaffolds

Mesh scaffolds can be either woven or non-woven fibrous materials that have high porosity, allowing for easy cell seeding (Chung and Burdick, 2008). One drawback to mesh scaffolds is that they are not suitable for immediate implantation because they lack the mechanical integrity required to withstand forces generated in joints. As a result, it is generally suggested that mesh scaffolds be pre-cultured *in vitro* before implantation

as a means of generating tissue that will enhance the mechanical properties of the scaffold, allowing it to withstand applied compressive and shear forces (Uematsu *et al.*, 2005).

2.3.2.2 Sponge scaffolds

Sponge scaffolds are materials that have defined pore sizes, which can be generated by several methods, including porogen leaching, freeze drying, and gas foaming (Chung and Burdick, 2008). Unlike the mesh structures previously described, sponge scaffolds may be more appropriate for direct implantation without the need for pre-culture due to their enhanced mechanical properties.

2.3.2.3 Hydrogel scaffolds

Synthetic hydrogels are a relatively new class of scaffolds being investigated for use as cartilage tissue engineering scaffolds. Most hydrogels being studied for tissue engineering scaffolds are composed of natural polymers, such as hyaluronic acid, collagen, and alginate. Hydrogels are composed of crosslinked hydrophilic polymers and thus swell in an aqueous environment. Advantages of using hydrogels as scaffolds include reduced protein interaction relative to other scaffold types, the ability to maintain shape upon swelling (Darling and Athanasiou, 2004), possessing similar water content to other tissues in the body, and the potential for easy cell incorporation. With respect to cell incorporation, cells may be seeded directly into a hydrogel by encapsulation as long as the environment in which the hydrogel is generated is nontoxic to them, allowing the cells to maintain their circular morphology. Maintaining circular morphology is an

important factor for chondrocyte phenotype maintenance (Kuo *et al*., 2006). In addition to cells, growth factors may also be encapsulated into hydrogels. This can be accomplished by either bathing hydrogels in growth factors following their formation, or by incorporating growth factors into a hydrogel before crosslinking, although significant denaturation can occur during the crosslinking reaction. Such incorporation further enhances the potential usefulness of hydrogels, allowing for stem cell differentiation in a gradient-free environment (Darling and Athanasiou, 2004). Additionally, hydrogels may be injected into spaces as polymers and crosslinked *in situ* as required, potentially allowing for a minimally invasive procedure for tissue repair (Drury and Mooney, 2003). One drawback to using hydrogels is that they are generally weak scaffold materials when physically crosslinked, limiting their potential for direct implantation into the body after formation. The mechanical strength of hydrogels can be increased by chemically crosslinking. However, crosslinking agents are generally quite cytotoxic, although some agents can maintain fairly high cell viability during the crosslinking process when used in low concentrations (Williams *et al.*, 2003).

2.3.3 Drug delivery

If tissue formation is to be achieved *in situ* following scaffold implantation, it is important to either recruit cells into the scaffold and/or ensure that sufficient tissue formation is achieved. One method by which this can be achieved is through drug delivery. A general drug delivery strategy involves the incorporation of a drug into a biomaterial, which allows for the slow release of the drug over time in a predictable manner. Commonly, a drug is incorporated directly into the scaffold where tissue

formation is desired. For example, a proliferative agent may also be incorporated into a scaffold in order to allow the cells within a scaffold to either generate significantly more repair tissue, or to generate tissue more quickly. Additionally, a chemotactic (cell-attracting) agent may be delivered from a scaffold over time to recruit target cells into the scaffold.

Chapter 3

Materials and Methods

3.1 Media supplement evaluation for cartilage tissue culture

One of the main goals of the present research is to elucidate the chemotactic effect that various growth factors may have on bovine articular chondrocytes. The most common supplement for cartilage and chondrocyte culture is fetal bovine serum (FBS), which contains trace amounts of various growth factors and other cytokines, and as such, potential alternatives to FBS were investigated.

3.1.1 Osteochondral explant harvest

Bovine osteochondral explants were harvested from the metacarpal-phalangeal joints of 18-24 month old calves of various species that were donated by Brian Quinn's Meats (Yarker, ON). Joints were opened under non-aseptic conditions and osteochondral explants were cored using a hand drill with 3/8" x ¼" super abrasive diamond coring bit (Starlite Industries Inc., Rosemont, PA, USA). Following coring, 8 mm diameter osteochondral explants were removed using the OATS^{TM} harvesting tools (Arthrex Inc, Naples, FL, USA). The harvested explants were placed in 20 mL Ham's F12 media (HyClone, Logan, UT, USA) supplemented with 25 mM HEPES (*N*-2-hydrocyethylpuperazine-*N'*-2-ethanesulfonic acid) (Sigma Aldrich Ltd, Oakville, ON), 300 U/mL penicillin, 300 µg/mL streptomycin, and 0.75 µg/mL amphotericin B

(Invitrogen Canada Inc, Burlington, ON). Immediately following harvest, the explants were washed under aseptic conditions with 20 mL Ham's F12 media supplemented with 25 mM HEPES, 100 U/mL penicillin, 100 ug/mL streptomycin, and 0.25 ug/mL amphotericin B (hereinafter referred to as 'standard media'). Following the wash, explants were transferred to a final Petri dish containing 20 mL standard media.

3.1.2 Osteochondral explant culture

The osteochondral explants were divided into groups containing standard media with 100 μg/mL (Sigma Aldrich Ltd) ascorbic acid and supplemented with one of the following: 1) 10% fetal bovine serum (FBS) (Sigma Aldrich Ltd), 2) 1X Nutridoma (Roche Diagnostics Canada, Laval, QC), 3) 1% Insulin-transferrin-sodium selenite (ITS), or 4) Control media containing no supplement. Each group contained 12 samples, and samples were cultured in 24-well culture plates with 1 mL of respective media. The explants were cultured in a sterile incubator at 37 ^{o}C and 95% relative humidity supplemented with 5% CO_2. Media was changed three times per week.

3.1.3 DNA, proteoglycan, and collagen synthesis

Twenty four hours prior to tissue harvest, samples were labeled with either 5 μCi of [3H]proline (PerkinElmer, Waltham, MA, USA), or with 5 μCi each of both [3H]thymidine (PerkinElmer) and [35S]sulfate (PerkinElmer). [^{3}H]proline was used to assess newly synthesized collagen within each sample (Peterkofsky and Diegelman, 1971), [^{3}H]thymidine was used to assess cell proliferation (Leblond *et al*., 1959), and [^{35}S]sulfate was used to assess newly synthesized proteoglycans (Bostrom and Mansson,

1952). Following 24h incubation with the respective radioisotope, each sample was washed 3 times in pH 7.4 phosphate buffered saline (PBS), blotted dry, and the cartilaginous portions of the explants were removed by use of a scalpel. Following papain digestion (§3.1.5), a 100 μL aliquot of each sample was mixed with 5 mL of scintillation fluid and read in a β-scintillation counter (LS6500 Multipurpose Scintillation Counter, Beckman Coulter Canada Inc, Mississauga, ON).

3.1.4 Tissue wet and dry mass determination

The wet weight of each sample was determined following harvest using a P-114 balance (Denver Instruments, Denver, CO, USA). Following wet weight determination, each sample was lyophilized (Fisher Scientific, Ottawa, ON) overnight and the dry weight of each sample was also determined.

3.1.5 Tissue digestion

In order to allow for radioisotope incorporation analysis and quantification of DNA, proteoglycans, and collagen content within each sample, all tissue samples were digested in 40 μg/mL papain (Sigma-Aldrich Ltd) in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid, and 2 mM dithiothreitol (Sigma-Aldrich Ltd) at 65^{o}C for 72 h. Samples were stored at -20^{o}C until they were analyzed.

3.1.6 DNA quantification

The amount of DNA in each sample was determined using the Hoechst 33258 dye binding assay and fluorometry (Kim *et al.*, 1988) for each papain-digested sample. Prior

to running the assay, samples were diluted in PBS, pH 7.4. The assay was carried out in 96-well fluorescence plates (VWR International, Mississauga, ON). A standard curve was generated using calf thymus DNA (Sigma-Aldrich Ltd). Samples reacted with the Hoechst 33258 dye (Sigma-Aldrich Ltd) and the ensuing fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Each sample and standard was measured in triplicate.

3.2 Chondrocyte chemotaxis in a modified Boyden chamber

3.2.1 Chondrocyte isolation

Bovine cartilage was harvested from the metacarpal-phalangeal joints of 18-24 month old calves that were donated by Brian Quinn's Meats (Yarker, ON). Joints were opened under aseptic conditions and cartilage was removed from the underlying bone with the use of a No. 11 scalpel blade. The harvested cartilage was placed in 15 mL standard media. The cartilage was digested in 0.5% (w/v) protease (Sigma-Aldrich Ltd) at 37^{o}C for 2h. The cartilage was then washed three times in standard media and was further digested in 0.1% collagenase A (Roche Diagnostics Canada) at 37^{o}C for 18h. Following collagenase treatment, the digested cartilage was passed through a 200 mesh filter (Sigma-Aldrich Ltd) to remove any pieces of undigested cartilage and any bone fragments. The filtered cell suspension was centrifuged at 600 rpm for 7 min, and the supernatant was aspirated off. The remaining cell pellet was then suspended in fresh standard media. This washing process was repeated three times. The cell viability was

assessed using the trypan blue (Sigma-Aldrich Ltd) dye exclusion method (Mishell and Shiigi, 1980).

3.2.2 Modified Boyden chamber preparation and cell seeding

Several agents known to induce chondrocyte chemotaxis were tested over a range of concentrations. The chemotactic agents investigated were IGF-I, bFGF, and PDGF-bb (Source: recombinant human) (Peprotech, Inc., Rocky Hill, NJ, USA) in concentrations ranging from 2.5 - 100 ng/mL. Chondrocyte chemotaxis was evaluated using a modified Boyden chamber (Corning Costar, Lowell, MA, USA). The upper and lower compartments of the chamber were separated by a 10 μm thick polycarbonate membrane with 8 μm pores. Prior to cell seeding, the upper and lower compartments were incubated in 0.1% BSA (cell culture tested, ≥96%) (Sigma-Aldrich Ltd) at 37^{o}C for 2 h to remove nonspecific protein binding sites. The lower compartments of the modified Boyden chambers were then filled with standard media supplemented with the chemotactic agent and 0.1% BSA. The upper compartments were loaded with 2.0×10^5 primary bovine articular chondrocytes suspended in standard media. After 24 h, chondrocytes remaining on the upper side of the membranes were removed using a cotton swab. Chondrocytes that migrated to the under side of the membrane were fixed in methanol for 5 min and stained with toluidine blue (Sigma-Aldrich Ltd). Chondrocyte migration was assessed by counting the number of chondrocytes in six random 0.5 mm^2 fields on the underside of the membrane using an inverted Hund Wetzlar light microscope (model Wilovert S) with a mounted Leica camera (model DFC320) and ImageJ software at 100X magnification.

The average cell counts on the underside of the membrane for each group were normalized to the average cell count on the underside of the membrane for the growth-factor-free control. This was done by dividing the average cell count for each group by the average cell count for the growth-factor-free control.

3.3 Chemotactic outgrowth of chondrocytes from bovine cartilage

3.3.1 Bovine cartilage explant harvest

Bovine cartilage explants were harvested from the metacarpal-phalangeal joints of 18-24 month old calves that were donated by Brian Quinn's Meats (Yarker, ON). Joints were opened under aseptic conditions and cartilage was removed from the underlying bone with the use of a No. 11 scalpel blade. The cartilage fragments that were removed from the bone were trimmed into squares with approximate dimensions of 2 mm x 2 mm x 1 mm. The trimmed explants were placed in 20 mL standard media.

3.3.2 Bovine cartilage explant culture

Explants were cultured in ITS-supplemented standard media in the presence of various concentrations of IGF-I, a known chemotactic agent for chondrocytes. The IGF-I concentrations ranged from 0 - 250 ng/mL. Explants were assigned to one of the concentration groups randomly.

3.3.3 Outgrowth assessment

Preliminary work showed that chondrocytes would begin outgrowing from the cut surface of the tissue (i.e., the periphery of the tissue) after 10 days in culture. Therefore, the number of chondrocytes outgrown from each sample was assessed every day between 10 and 20 days in culture. An outgrown cell was defined as a cell located outside of the cut surface of the tissue, as illustrated in Figure 3.1. The outgrowth was assessed using a light microscope at 100x magnification.

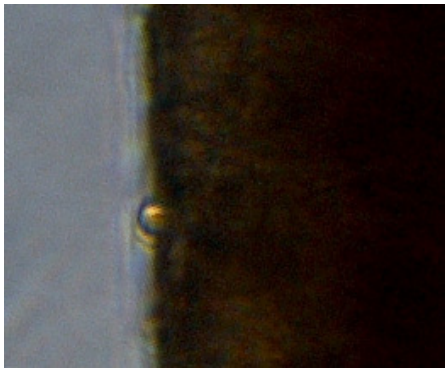

Figure 3.1. Example of an outgrown chondrocyte; from the cut surface of a cartilage explant.

3.4 Collagenase penetration into cartilage explant tissue

Cartilage explants were harvested as previously outlined (§ 3.3.1). Explants were treated with various concentrations of collagenase A (source: *Clostridium histolyticum*) (Roche Diagnostics) (0.15% - 0.6%) for various amounts of time (2.5 – 60 min). Following treatment, the samples were washed in PBS and fixed in 4% paraformaldehyde. The samples were then prepared for histology by replacing paraformaldehyde with 100% ethanol through a graded series of washes. Each sample

was embedded in paraffin, cut into 10 μm thick sections, and stained with picrosirius red. Each sample was viewed under light microscopy at 40X magnification, and the depth of the collagenase penetration into the tissue was determined by measuring the distance from the periphery of the tissue to the region of degraded collagen within the tissue. Regions in the explants that had been degraded by the collagenase stained more intensely than non-degraded regions.

3.5 Collagenase digestion and chondrocyte outgrowth from cartilage explant tissue

Previous studies have shown that collagenase can be used to promote chondrocyte outgrowth from cartilage explants (Qiu *et al.*, 2000). Bovine cartilage explant tissue was obtained as outlined previously (§3.3.1). Explants were digested 0.15% collagenase (Roche Diagnostics Canada) for various amounts of time 5 min. Following digestion, each explant was washed thoroughly 3 times in standard media. Explants were cultured as outlined previously (§3.3.2). Each explant was assessed for outgrowth (§3.3.3) each day beginning on day 1 of the culture period, as outlined previously.

3.6 Chondrocyte repopulation of the zone of death

Bovine cartilage explants were harvested as outlined previously (§3.5). Cylindrical tissue samples were obtained using 8 mm biopsy punches (Miltex Inc., York, PA, USA). An inner core of 3 mm diameter was punched out of each 8 mm diameter cylindrical explant using a dulled biopsy known to induce a ZCD. A device was designed that ensured that each inner core was punched out of the exact centre of each sample. Each inner core and corresponding outer ring were assigned to one of the following

groups: 1) Treatment with 0.6% collagenase for 10 min, 2) Treatment with 0.6% collagenase for 10 min and supplementation with an 25 ng/mL of: i) PDGF-bb, ii) IGF-I, or iii) bFGF, 3) Control group with no treatment, or 4) Control group with no treatment to be cultured for 10 days for determination of the initial ZCD. Following treatment, each inner core was re-implanted within its respective outer ring (as illustrated in Figure 3.2) and each sample was cultured for 4 weeks, with the exception of the group cultured for 10 days (3.1.2). Following the culture period, samples were assessed for integration of inner core and outer ring via mechanical testing, histological properties of the integration region between the inner core and outer ring, or the extent of chondrocyte migration through the ZCD via LIVE/DEAD^{TM} staining (§3.6.1).

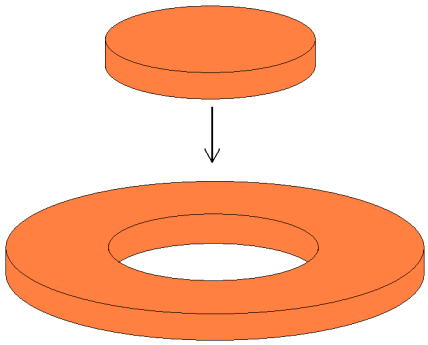

Figure 3.2. A 3 mm inner core was punched out of an 8 mm cartilage explant. The inner core of the cartilage explant tissue was re-implanted into the outer ring following treatment with collagenase.

3.6.1 Zone of chondrocyte death measurement

In addition to the groups given in Cartilage explants were harvested as previously outlined (§3.3.1). Following tissue harvest, cylindrical pieces of cartilage were obtained

using the OATS^{TM} system, fresh biopsy punches, and biopsy punches dulled with aid of a wooden block. The cylindrical pieces of cartilage were cultured for 10 days (§3.1.2) in order to allow the ZCD to fully develop (Huntley *et al.*, 2005). Following the culture period, each sample was stained using the LIVE/DEAD^{TM} staining kit (Molecular Probes). The LIVE/DEAD^{TM} kit contains calcein AM and ethidium homodimer 1. Live cells, unlike dead cells, have intracellular esterase activity, which causes the conversion of nonfluorescent calcein AM to fluorescent calcein, which is visible at an excitation/emission wavelength of ~495/~515 nm. Dead cells have damaged membranes through which ethidium homodimer 1 can enter into cells, and following binding with nucleic acids, the ethidium homodimer 1 undergoes a 40-fold increase in fluorescence, which is visible at an exitation/emission wavelength of ~495/~635 nm. Samples imaging was done using a Leica TCS SP2 multiphoton confocal inverted microscope with Leica Image Pro Plus software at 40× magnification. The extent of the ZCD was determined by measuring the distance from the periphery of the tissue to the inner region of the tissue containing live chondrocytes. Ten measurements were made for each image obtained at approximately 50 μm increments along the tissue periphery. An example of a sample measurement is illustrated in Figure 3.3. The ZCD measures differ from the previous measures of chondrocyte outgrowth. As live chondrocytes move toward to the periphery of the tissue, they repopulate the ZCD, thereby decreasing the extent of the ZCD.

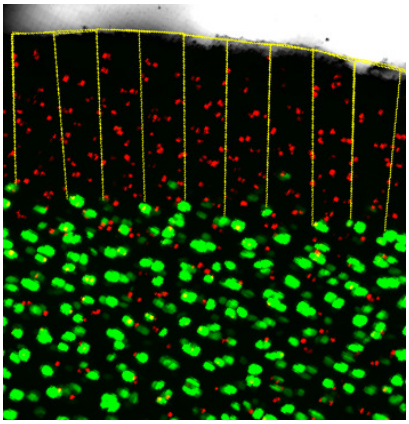

Figure 3.3. Representation of measurement of the zone of chondrocyte death. The extent of the zone of chondrocyte death was determined by measuring the distance from the tissue periphery to the inner region of live cells at several points around each sample.

3.7 Chondrocyte chemotaxis through methacrylated glycol chitosan hydrogel

Chemotaxis experiments were conducted in order to determine the extent to which chondrocytes could migrate into methacrylated glycol chitosan (mGC).

3.7.1 Methacrylated glycol chitosan gel preparation

Methacrylated glycol chitosan (6% degree of *N*-methacrylation as determined by ^{1}H NMR) was synthesized and generously donated by Ms. Abby Sukarto. mGC was dissolved on a shaker overnight in PBS, pH 7.4 to a concentration of 60 mg/mL. The photoinitiator, 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) (Sigma Aldrich Ltd), was dissolved to a concentration of 10 mg/mL in PBS. The mGC

and Irgacure solutions were mixed in a 9:1 ratio, respectively, and the resulting solution was mixed on a shaker to ensure homogeneity of the mixture.

3.7.2 Photocrosslinking the hydrogel

170 uL of the mGC/Irgacure mixture (§3.7.1) was added to cell culture filter inserts (Millicell CM®, Millipore Corp, Bedford, MA, USA) and photocrosslinked using an EXFO Lite lamp (EXFO Toronto, Mississauga, ON) with EFOS UV filter (320-480 nm, EFOS Inc, Mississauga, ON) at an intensity of 10 mW/cm^2 for 90 s. The cell culture filter inserts containing the photocrosslinked hydrogels were washed three times over a period of one hour in standard media containing 1% ITS.

3.7.3 Chondrocyte chemotaxis through hydrogel

After washing the photocrosslinked mGC in standard media, the inserts were placed in a tissue culture plate, where either 400 uL of standard media containing 1% ITS or standard media containing 1% ITS supplemented with 50 ng/mL IGF-I was added to the outside of the filter. Primary chondrocytes, harvested as outlined previously (§3.2.1), were seeded on top of the photocrosslinked mGC samples at a density of 2.0×10^5 chondrocytes per hydrogel suspended in 300 uL of standard media containing 1% ITS. The extent of chondrocyte migration into the mGC for up to one week in culture was assessed using LIVE/DEAD staining and by scanning through the depth of the gel using a Leica TCS SP2 multiphoton confocal inverted microscope with Leica Image Pro Plus software (§3.6.1).

3.8 Statistical analyses

For each experiment involving isolated chondrocytes, the chondrocytes were obtained from a pooled source of dissected legs to reduce variability in the samples. For each experiment involving cartilage explant tissue, explant samples were randomized between experimental groups. All numerical results are expressed as a mean ± standard error of the mean (SEM). Experimental and control groups from different conditions for relevant experiments were statistically compared using one-way ANOVA's with comparisons between individual groups assessed using Bonferroni-corrected t-tests. In all tests, significance was associated with $p < 0.05$.

Chapter 4

Results

4.1 Comparing various media supplements for cartilage tissue culture

The first experiment investigated various media supplements (ITS and Nutridoma) and their ability to maintain healthy cartilage tissue *in vitro* in the absence of serum. Bovine osteochondral samples were cultured for 10 or 20 days (n=9 per group per time period) and various indices of overall tissue health were assessed, including DNA and ECM synthesis (Figure 4.1 and Figure 4.2) and DNA content (Figure 4.3), and compared relative to samples cultured in 10% FBS. Three samples were lost due to infection by day 10 (two samples from the control group and one sample from the group supplemented with FBS).

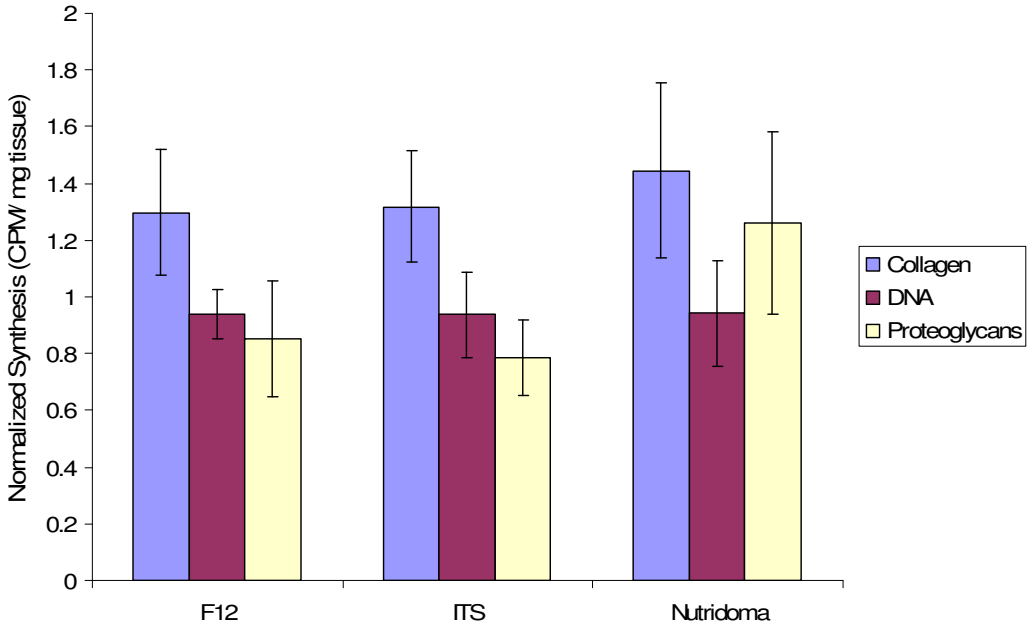

Figure 4.1. Normalized synthesis rates for bovine articular cartilage cultured in various media supplements after 10 days in culture. Synthesis rates were normalized to controls cultured in 10% FBS (n=9, Mean ± SEM).

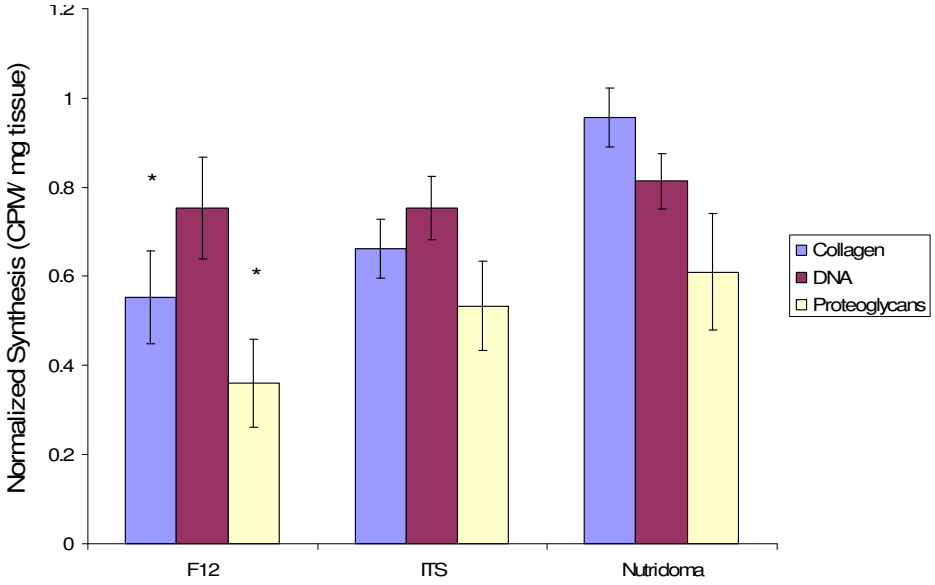

Figure 4.2. Normalized synthesis rates for bovine articular cartilage cultured in various media supplements after 20 days in culture. Synthesis rates were normalized to controls cultured in 10% FBS (n=9, Mean ± SEM, * indicates $p < 0.05$).

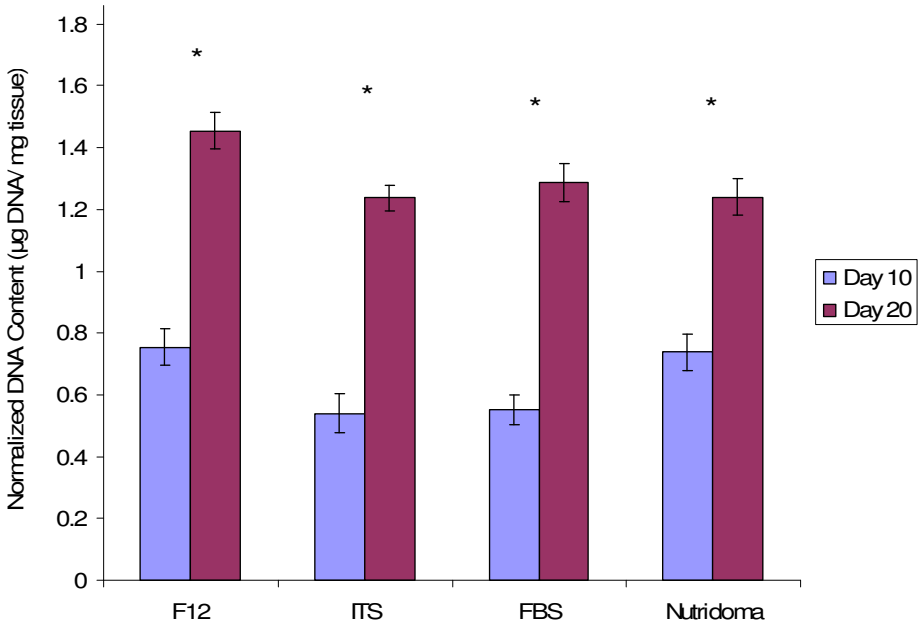

Figure 4.3. DNA content in bovine articular cartilage normalized to tissue dry mass in various media supplements after 10 and 20 days in culture (n=9, Mean ± SEM, * indicates $p < 0.05$ between day 10 and day 20 for a given media supplement).

The media supplements were evaluated relative to FBS, which is considered to be the gold standard supplement in cell culture. FBS resulted in significantly ($p < 0.05$) higher collagen and proteoglycan production relative to the F-12 control group after 20 days in culture (Figure 4.2). No other significant differences were observed between the remaining groups at either time point in terms of synthesis rates and DNA content. Each media supplement did result in significantly ($p < 0.05$) increased DNA content from day 10 to day 20 in culture (Figure 4.3).

4.2 Five week culture in ITS-supplemented media

Bovine cartilage was cultured in ITS-supplemented media for a longer period of time (up to 5 weeks) in order to establish how long healthy cartilage can be grown *in vitro*. Following the culture period, various indices of overall tissue health were assessed, including DNA and ECM synthesis and DNA content. Results suggested that there was a trend whereby ECM synthesis rates were reduced beyond 1 week in culture, and remained low throughout the experiment (Figure 4.4). However, there was no apparent decrease in rate of DNA synthesis throughout the duration of the culture period. Additionally, DNA content appeared to increase with culture time throughout the 5 week culture period (Figure 4.5).

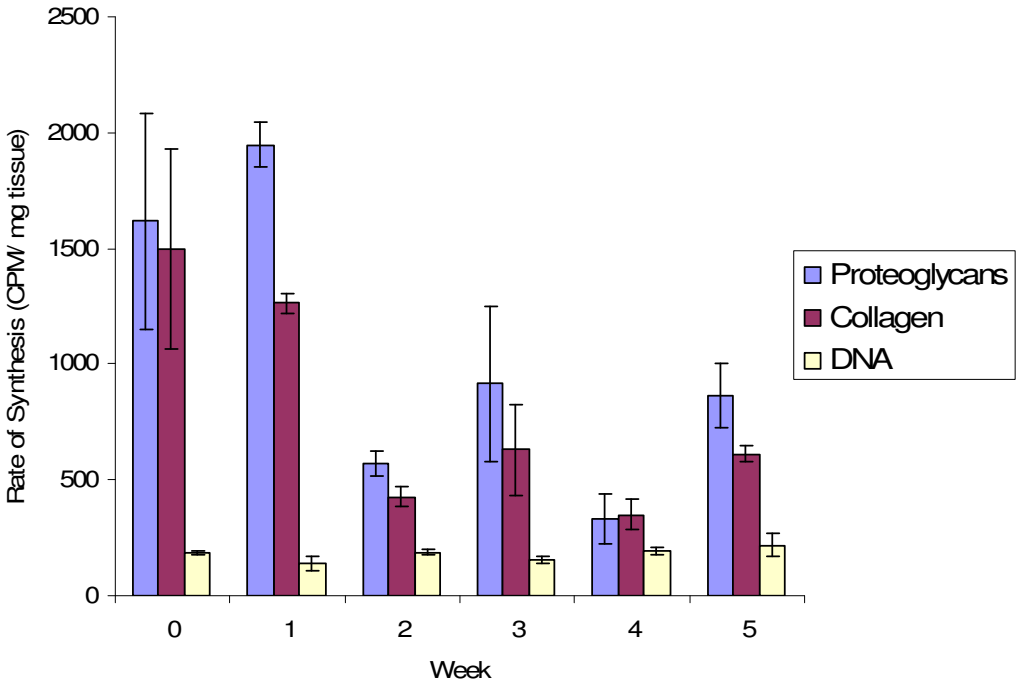

Figure 4.4. Synthesis rates for bovine articular cartilage cultured in ITS-supplemented media for up to 5 weeks in culture. (n=3, Mean ± SEM).

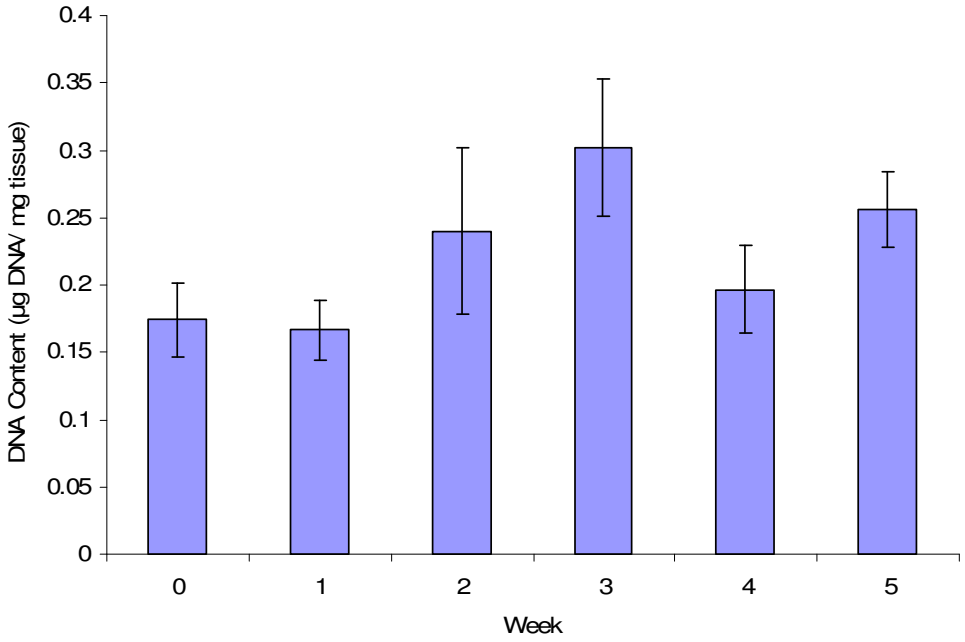

Figure 4.5. DNA content for bovine articular cartilage cultured in ITS-supplemented media for up to 5 weeks in culture. (n=6, Mean ± SEM).

4.3 Chondrocyte chemotaxis in a modified Boyden chamber

A commonly used method for determining cell chemotaxis is a modified Boyden chamber. Several agents (IGF-I, PDGF-bb, and bFGF) that induce chondrocyte chemotaxis were assessed over a range of concentrations in modified Boyden chambers in order to determine optimal concentrations for inducing chemotaxis. The cell counts obtained for each concentration of each growth factor were normalized to the growth factor-free controls. There was a general trend whereby none of the growth factors examined were able to induce chondrocyte chemotaxis at concentrations up to 10 ng/mL. However, there was a general increase in chondrocyte chemotaxis for each of the growth

factors at concentrations of 25, 50, and 100 ng/mL. There was no obvious difference between the growth factors and their ability to induce chondrocyte chemotaxis.

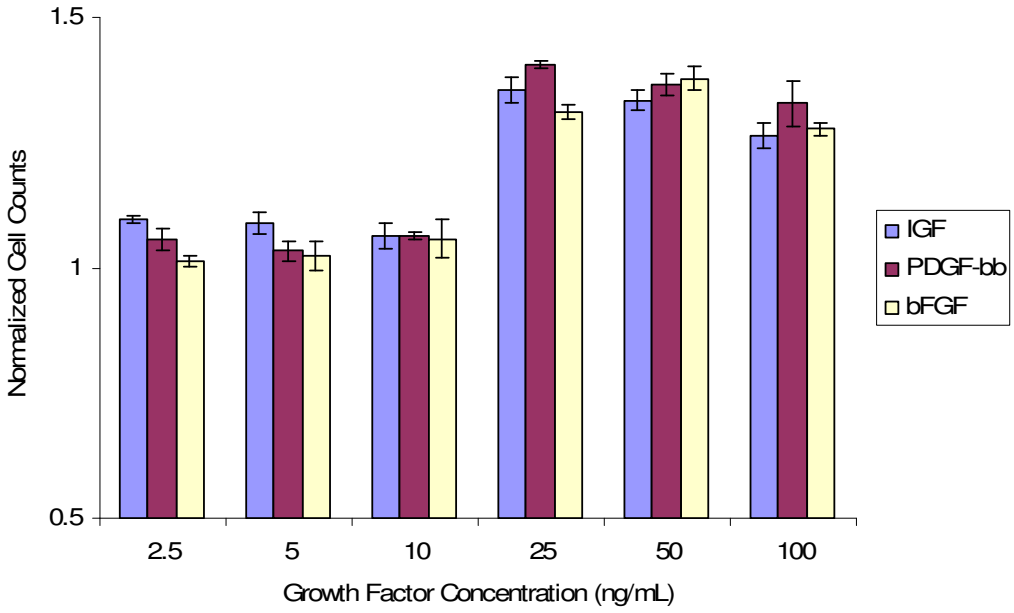

Figure 4.6. Chondrocyte counts on the underside of the Boyden Chamber membrane for various concentrations of IGF-I, PDGF-bb, and bFGF normalized to growth-factor-free controls.

4.4 Chemotactic outgrowth of chondrocytes from native cartilage

The ability of chondrocytes to grow out of native tissue to the tissue periphery was tested with the aid of chemotactic agents. Initial experiments indicated that the minimum amount of time required for chondrocyte outgrowth was approximately 10 days, and therefore, the number of outgrown chondrocytes between day 10 and day 21 in culture was determined. The total number of outgrown chondrocytes remained low

throughout the experiment, as indicated in Figure 4.7. There was no significant effect of time on the number of outgrown cells, although there was a significant effect of IGF-I concentration ($p < 0.001$). Thus, the number of outgrown cells for all time points were grouped for each IGF-I concentration (Figure 4.8), and Bonferroni-corrected t-tests were used to determine which IGF-I concentration was significant relative to control. The only IGF-I concentration that significantly increased chondrocyte outgrowth relative to control groups was 25 ng/mL. Although the other concentrations were not statistically significant, there was a general trend whereby the amount of chondrocyte outgrowth decreased from 25 ng/mL through 250 ng/mL, as is illustrated in Figure 4.8

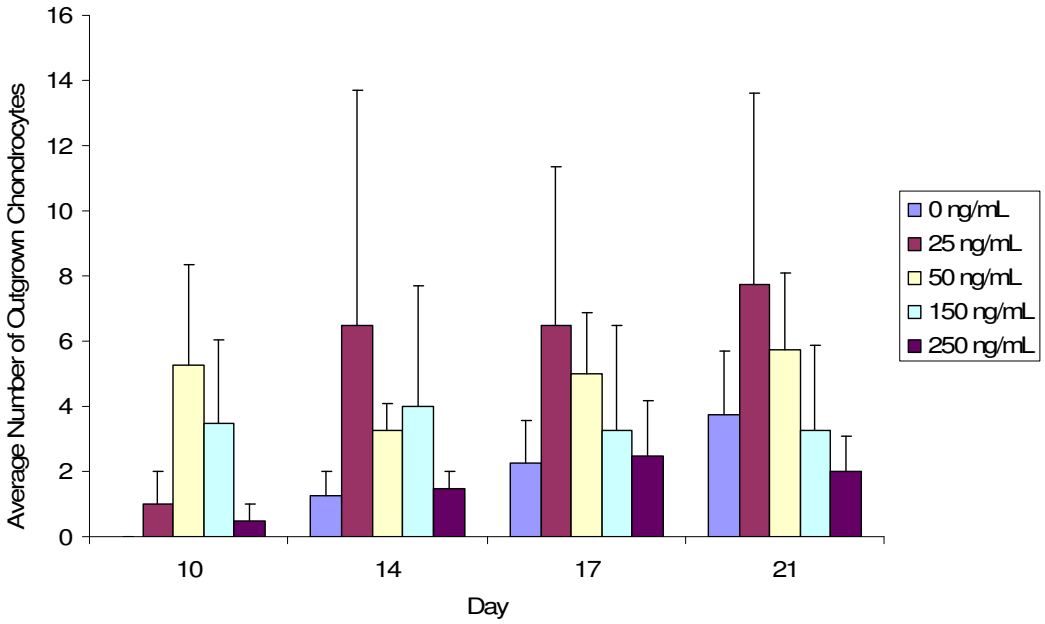

Figure 4.7. Chondrocyte outgrowth from native bovine articular cartilage in the presence of various concentrations of IGF-I, a known chemotactic agent (n=4, Mean ± SEM).

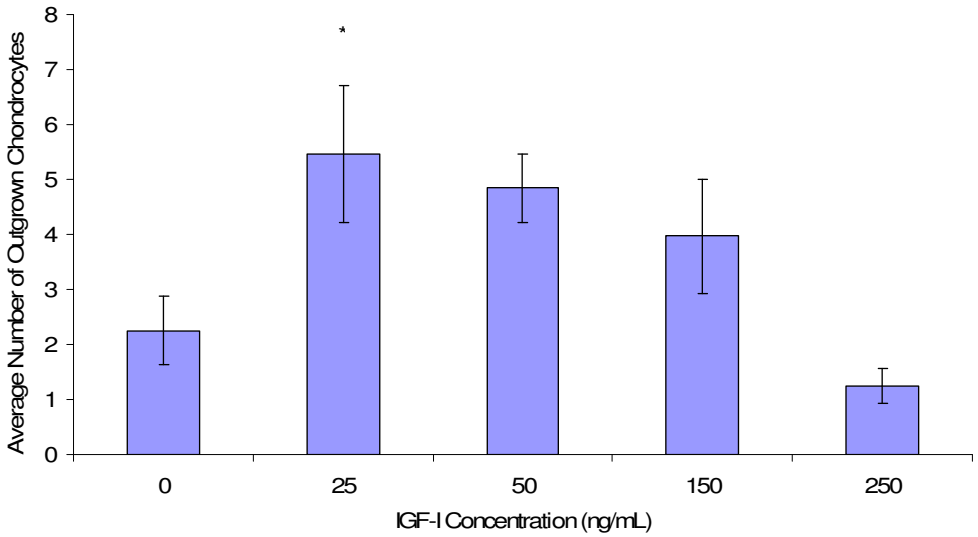

Figure 4.8. Chondrocyte outgrowth from native bovine articular cartilage in the presence of various concentrations of IGF-I. The average number is taken from all time points at which cell outgrowth was observed from day 10 to day 21 in culture. (n=32, Mean ± SEM, * indicates $p < 0.05$).

Although there was a statistically significant effect of IGF-I concentration on chondrocyte outgrowth from native cartilage, the number of chondrocytes actually outgrowing was quite small, despite the fact that each sample had approximately 8 mm^2 of surface area from which chondrocytes could outgrow.

4.5 Collagenase penetration into cartilage explant tissue

The extent of collagenase penetration into the tissue was not found to be dependent on distance from the articular surface in samples harvested using the OATS osteochondral harvesting system (Figure 4.9), whereas the extent of collagenase

penetration into the tissue is dependent on distance from the articular surface in samples harvested using a sharp biopsy punch (Figure 4.9). Following tissue harvest with the OATS osteochondral transfer system, longer collagenase treatment times and higher collagenase treatment concentrations generally resulted in greater penetration into the cartilage. Following tissue harvest with a sharp biopsy punch, longer collagenase treatment times and higher collagenase concentrations generally resulted in greater penetration into the cartilage near the articular surface, but not in the deeper regions of cartilage.

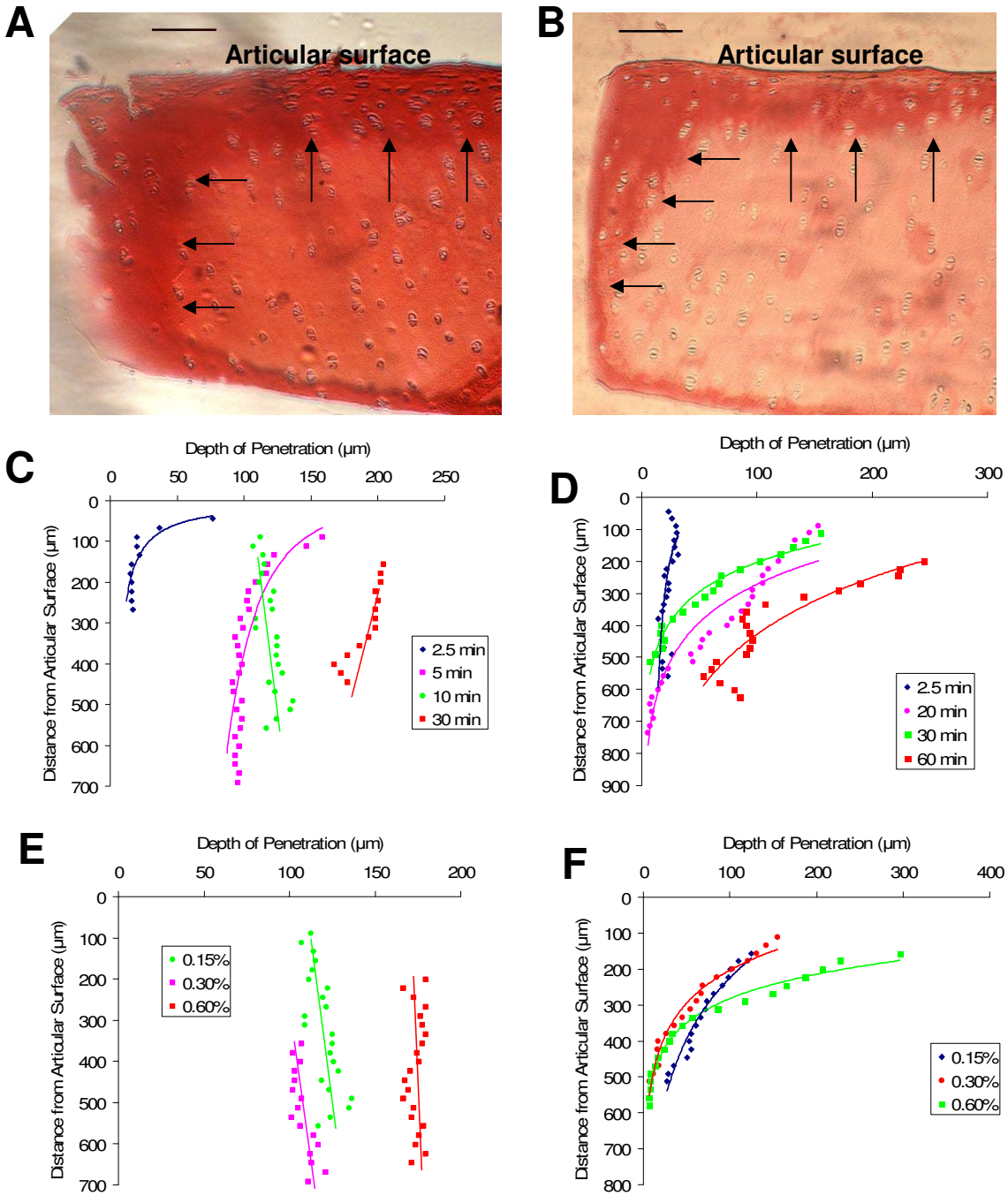

Figure 4.9. Collagenase penetration into bovine articular cartilage harvested using either the OATS osteochondral transfer system (A, C, E), or a sharp biopsy punch (B, D, F). In sections stained with picrosirius red (A, B), the more intensely-stained regions (indicated by arrows) indicate the depth to which collagenase penetrated into the tissue following 30 min treatment with 0.15% collagenase. Magnification 40×, scale bar = 100 µm.

4.6 Collagenase digestion and chondrocyte outgrowth from native cartilage

Collagenase has been used in the past to allow for chondrocyte migration through native cartilage, and as such, was investigated for its potential to allow for enhanced chondrocyte chemotaxis through native cartilage. Chondrocyte outgrowth was assessed between day 1 and day 21 in cultures treated with 0.15% collagenase for 5 min and supplemented with or without 25 ng/mL IGF-I. Supplementing bovine articular cartilage explants with 25 ng/mL IGF-I after treating with 0.15% collagenase for 5 min resulted in significantly more chondrocyte outgrowth relative to controls not supplemented with IGF-I throughout 21 days in culture, as indicated by ANOVA ($p < 0.05$) (Figure 4.10). In the control group after 8 days in culture, there was also a marked decrease in the number of outgrown chondrocytes, possibly due to cell death and detachment from the tissue. Thus, collagenase treatment was a method that could be combined with chemotaxis to potentially promote chondrocyte outgrowth from native cartilage.

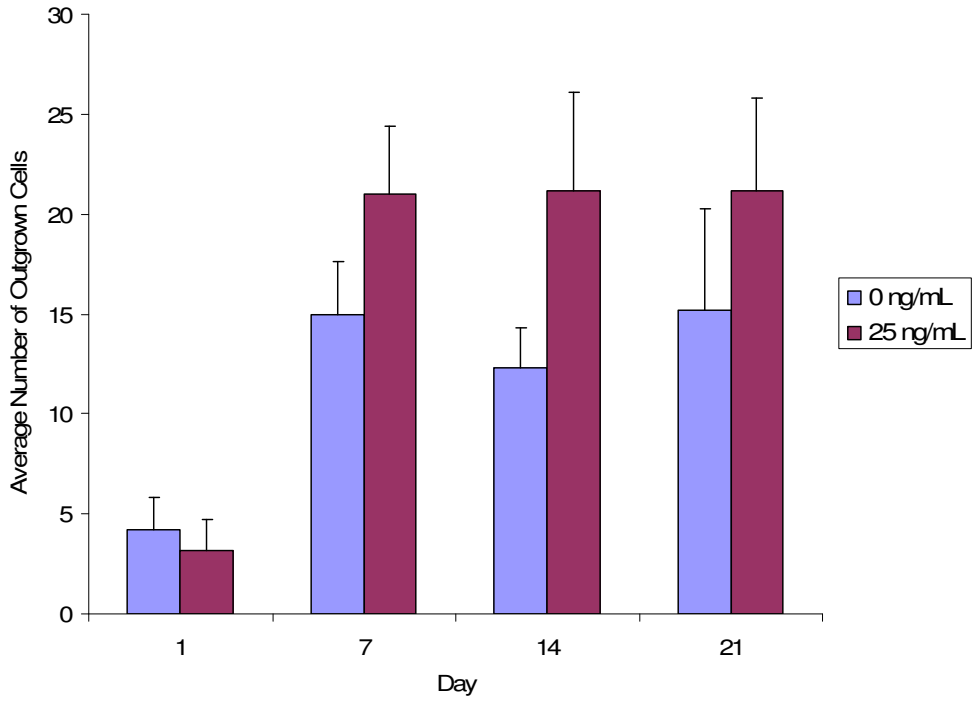

Figure 4.10. Average number of outgrown chondrocytes from bovine articular cartilage explants with and without supplementation with 25 ng/mL IGF-I after treatment with 0.15% collagenase for 5 min. The average number is taken from observations made between day 1 and day 21 in culture (n=6, Mean ± SEM).

Comparing the collagenase-treated samples with untreated samples illustrates the effect of collagenase alone in promoting chondrocyte outgrowth. Treating bovine articular cartilage explants with 0.15% collagenase for 5 min significantly ($p<0.05$) increased the number of outgrown chondrocytes relative to untreated controls between 13 and 17 days in culture (Figure 4.11). However, beyond 17 days, there was no statistically significant difference between the groups.

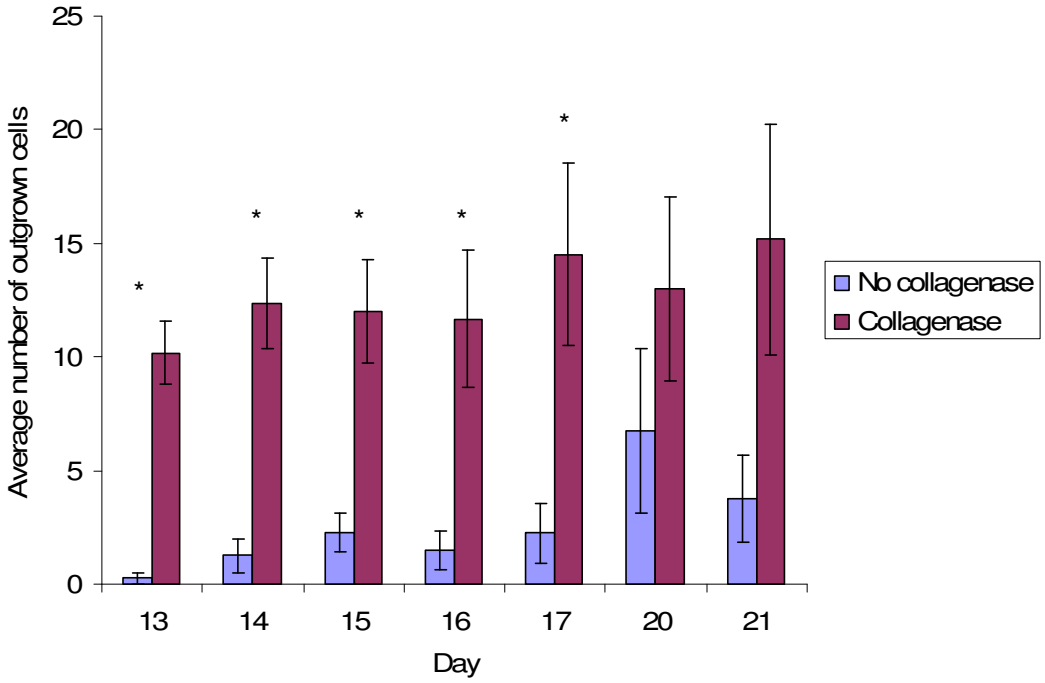

Figure 4.11. Average number of outgrown chondrocytes from bovine articular cartilage explants with and without treatment with 0.15% collagenase for 5 min. There was significantly more chondrocyte outgrowth in the collagenase-treated group between day 13 and day 17 in culture. (n=4 for non-collagenase treated group, n=6 for collagenase treated group, Mean ± SEM, * indicates $p < 0.05$ between collagenase-treated groups and controls at a given day).

4.7 Chondrocyte repopulation of the zone of chondrocyte death

An initial pilot experiment looking at the effect of IGF-I supplementation and treatment with 0.3% collagenase for 10 min was performed to determine the effect such treatments would have on the ZCD (§0). The results indicated that there was a significant effect of the various culture conditions on the ZCD ($p < 0.001$). Collagenase treatment reduced the ZCD by approximately 40% compared to the control (*collagenase:* 140 ± 17 µm; n=8, *control:* 241 ± 21; n=7). Although IGF-1 supplementation alone had no

discernible effect on the ZCD, supplementation with an average of 25 ng/mL IGF-1 after pre-treatment with collagenase significantly reduced the ZCD by 80% compared to the control (*IGF-1:* 255 ± 13 μm, n=11; *collagenase + IGF-1:* 48 ± 13 μm, n=12). In addition, there appeared to be a linear trend in the collagenase + IGF group whereby the ZCD decreased over time (*Day 7*: 65 ± 18 μm; *Day 21*: 34 ± 9 μm).

The results of the initial pilot study provided guidelines for the experiments detailed previously (§3.2 and §3.4). Therefore, the results of the chondrocyte chemotaxis experiments performed in a modified Boyden chamber were coupled with the results from the extent of collagenase penetration into the side of a cartilage explant to devise a treatment where chondrocyte migration into the ZCD could be optimized. Each of the chemotactic growth factors induced chondrocyte chemotaxis in modified Boyden chambers at concentrations of 25, 50, and 100 ng/mL. Additionally, IGF-I supplementation at 25 ng/mL was able to induce significant outgrowth from normal and collagenase-treated explant cartilage. Thus, each growth factor was supplemented at 25 ng/mL in all subsequent experiments. Furthermore, initial experiments using dulled biopsy punches indicated that the ZCD induced by the punches was approximately 175 μm. Therefore, tissue samples were treated with 0.6% collagenase for 10 min, as this treatment was found in a preliminary study to penetrate approximately 180 μm into the side of cartilage explants, and this should allow for ECM breakdown within the ZCD that is generated by the dulled biopsy punches.

Previous studies have shown that it can take up to 10 days for a ZCD to fully form (Tew *et al.*, 2000). Thus, the ZCD induced by explant harvest was determined by

culturing a separate group of explants for 10 days. Each of the treatment groups had a significantly reduced ZCD relative to the control group ($p<0.05$). Treatment with 0.6% collagenase for 10 minutes and supplementation with 25 ng/mL IGF-I resulted in a significantly reduced ZCD relative to each of the remaining treatment groups ($p < 0.05$) (Figure 4.12 and Figure 4.13). The initial explant harvest resulted in a ZCD of 179 ± 15 μm after 10 days in culture, whereas the control group had a ZCD 175 ± 13 μm, indicating that virtually no reduction in ZCD can be achieved without treatment over the course of the experiment. There was no significant difference between the collagenase treatment and treatment with either 25 ng/mL bFGF or 25 ng/mL PDGF-bb.

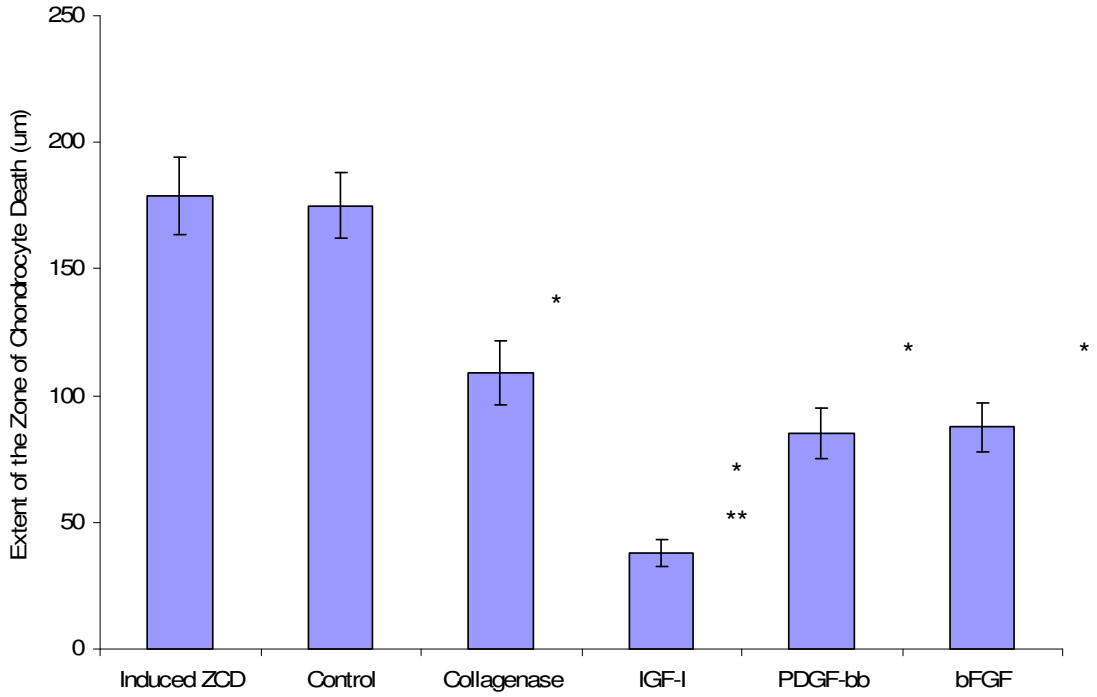

Figure 4.12. Zone of chondrocyte death (ZCD) following a 10 minute treatment with 0.6% collagenase, or collagenase + a chemotactic growth factor at 25 ng/mL. The induced ZCD is the extent of the ZCD after allowing it to develop. Cartilage explants were cultured for 4 weeks and ZCD was determined by measuring the distance from live chondrocytes within the tissue to the tissue periphery. (n=5, Mean ± SEM, * indicates $p < 0.05$ relative to control group, ** indicates $p < 0.05$ relative to collagenase-treated group).

One outcome measure that was going to be used to assess the extent of healing in the re-assembled cartilage explants was the amount of force required to separate the two pieces of cartilage from each other. However, upon the termination of the experiment, the inner piece of cartilage consistently fell out of the outer ring of cartilage, indicating an absence of healing. This finding was supported by histological assessment of several samples (data not shown).

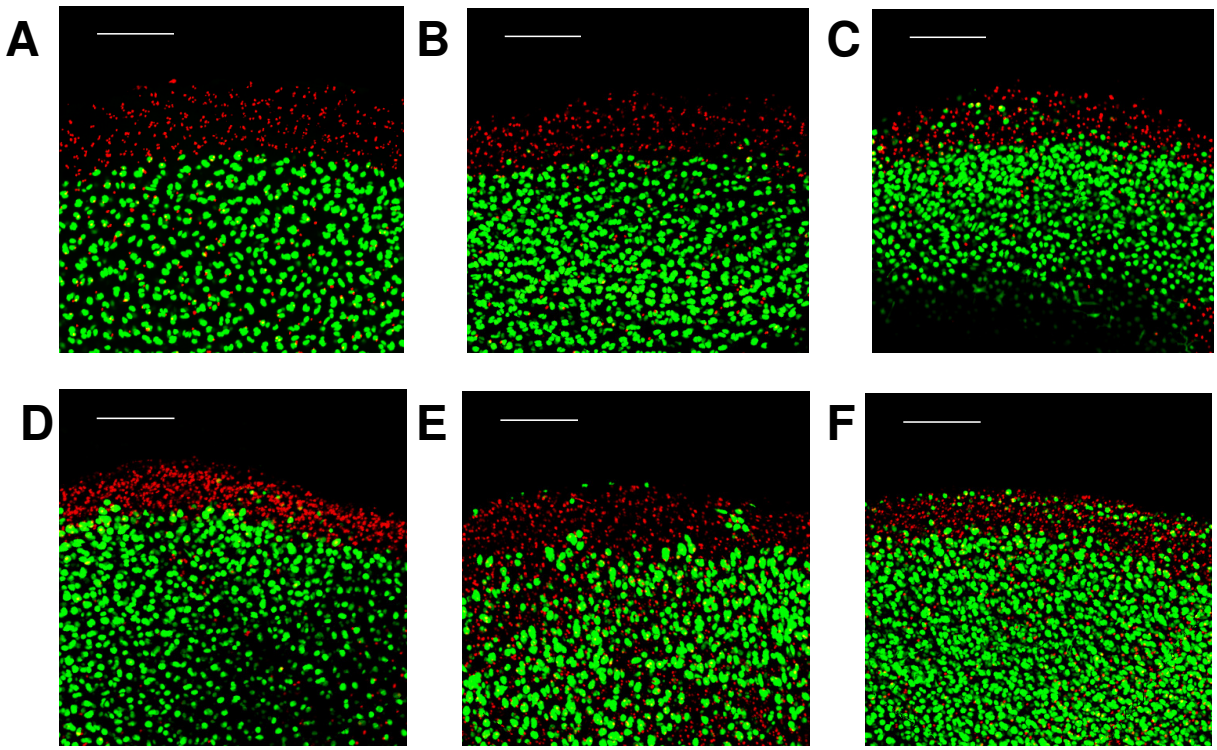

Figure 4.13. The effect of various treatments on the zone of chondrocyte death. Cells were stained with the LIVE/DEAD staining kit (live cells appear green, dead cells appear red) and viewed under confocal microscopy from the articular surface downward at 40× magnification. The initial zone of death induced by a biopsy punch (A) was not significantly different than the control group (B) after four weeks in culture. Treatment with 0.6% collagenase for 10 min (C) significantly reduced the zone of chondrocyte death relative to the control group, as did treatment with 0.6% collagenase for 10 min and supplementation with 25 ng/mL of either PDGF-bb (D) or bFGF (E). Treatment with 0.6% collagenase for 10 min and supplementation with 25 ng/mL IGF-I significantly reduced the zone of chondrocyte death relative to both the control group and the group treated with 0.6% collagenase for 10 min. Scale bar = 200 μm.

The cellularity within the ZCD region was also assessed in addition to the extent of the ZCD. However, in order to make appropriate comparisons between groups, the cellularity was normalized to the extent of the ZCD following the four week culture

period. The only significant difference between any of the groups was between the IGF-I treatment group and the PDGF-bb treatment group ($p < 0.05$) (Figure 4.14). This result indicates that there was a significantly greater number of cells within the ZCD region of the PDGF-bb region relative to the IGF-I region.

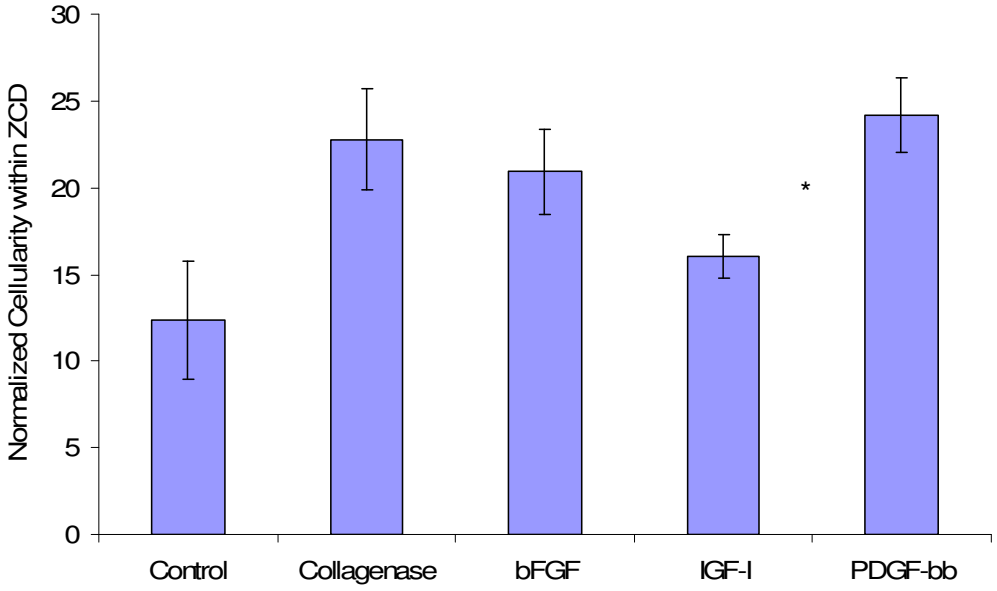

Figure 4.14. Cellularity within the ZCD region for each treatment group after 4 weeks in culture. The cellularity was normalized to the extent of the ZCD for each group after 4 weeks in culture. (n=5, Mean ± SEM. * indicates $p < 0.05$ relative to PDGF-bb).

4.8 Chondrocyte chemotaxis through methacrylated glycol chitosan

Chondrocytes were unable to migrate through the methacrylated glycol chitosan hydrogels. While dead chondrocytes were found on the bottom of the mGC hydrogels, the cells were found not to have migrated through the gel. At all time points, there were no cells found within any of the hydrogels. In fact, even when additional time points (4 h,

12 h) were added, there were still no chondrocytes found with any gel, although there were cells at the bottom of some samples at these time points. Additionally, cells that were originally seeded on top of the mGC died within a few days. Therefore, there was no evidence found that chondrocytes were able to migrate into the mGC, or even survive on the surface of the mGC under the conditions examined. Nonetheless, in separate experiments in which chondrocytes were seeded within the mGC hydrogels, the chondrocytes were able to maintain viability levels that have been found previously (A. Sukarto, personal communication, 2009).

Chapter 5

Discussion

5.1 Media supplement evaluation

Of the serum substitutes investigated, there was no significant difference between FBS and either ITS or Nutridoma in terms of ECM or DNA synthesis rates or DNA accumulation. A previous study comparing FBS and ITS supplementation in chondrocyte-seeded agarose hydrogels found that there was no significant difference in [^{3}H]proline incorporation throughout a 5 week culture period, although ITS-supplemented groups did have increased [^{35}S]sulfate incorporation at three, four, and five weeks in culture (Kisiday *et al.*, 2005). Additionally, there was no difference in cell division between ITS and FBS supplemented groups after the five week culture period, although ITS-supplemented cultures had slightly reduced cellularity after 6 and 14 days in culture, but not after 10 days in culture. No known studies have looked into comparing Nutridoma with FBS in the concentrations used in the present study, but one study did investigate Nutridoma at twice the concentration used in the present study (Glowacki *et al.,* 2005), as well as a different formulation of ITS than was used in the present study (ITS^{+3}), in porous collagen sponges. In this study, it was reported that Nutridoma-supplemented groups had no significant difference in cellularity after four days in culture from groups supplemented with 10% FBS, but ITS^{+3} groups did have reduced cellularity after four days in culture.

ITS was chosen for further investigation as an appropriate supplement because of its history of use in cartilage and chondrocyte culture (Kisiday *et al.*, 2005; Rotter *et al.*, 2005; Gigout *et al.*, 2005; Keenan *et al.*, 2006) and because Nutridoma has not been widely used in either cartilage or chondrocyte culture. While Nutridoma is a biochemically defined serum-free supplement, its exact formulation is proprietary, containing human proteins, cytokines, and organic and inorganic materials, whereas ITS has a known, defined composition.

5.2 Five week culture in ITS-supplemented media

Cartilage cultured in ITS-supplemented media had ECM synthesis rates that were significantly reduced after two weeks in culture. One implication of this finding is that the reduced ECM synthesis rates suggest that cartilage healing may not be attainable with ITS-supplemented media, as ECM production is required to allow for the cartilage integration (van de Breevaart Bravenboer *et al.*, 2004). The low ECM synthesis rates could have lead to decreased tissue production when testing the presence of cartilage integration following chondrocyte migration into the ZCD (§3.6). While chondrocytes were able to grow into the ZCD, and in some cases, to the periphery of the tissue, there was no evidence of cartilage healing following histological evaluation of the tissues. This could have been caused by an inability of the chondrocytes to produce significant amounts of tissue in the absence of a better media supplement.

While ECM synthesis was reduced after two weeks in culture, DNA content and synthesis rates were unaffected throughout the duration of the five week culture period.

This result is important when considering that one of the main goals of the present research is to promote chondrocyte migration through a ZCD. The chondrocytes are able to remain viable while being supplemented with ITS, and are able to replicate as well. As such, while ITS may not be suited to cartilage and chondrocyte culture in instances where significant tissue production is the end goal, it is suitable for the requirements of the present study in that maintaining chondrocyte viability is of importance.

5.3 Chondrocyte chemotaxis in a modified Boyden chamber

No known study has systematically looked at which concentrations of chemotactic agents induce chondrocyte chemotaxis. In the past, chondrocyte chemotaxis studies have looked at only high and low concentration responses to chemotactic agents (Mishima and Lotz, 2008), a single concentration response (Chang *et al.*, 2003), or did not use concentrations high enough to elicit a response (Hidaka *et al.*, 2006; Maniwa *et al.*, 2001). In terms of IGF-I, a previous study found that 10 ng/mL IGF-I had elicited an approximately two-fold increase in bovine chondrocyte chemotaxis relative to controls (Chang *et al.,* 2003). In the present study, there was no chemotactic effect of IGF-I at 10 ng/mL. One difference that could account for the discrepancy between the work of Chang *et al.* (2003) and the present study may be related to fact that in the present study, primary bovine articular chondrocytes were used, whereas Chang *et al.* (2003) used passaged chondrocytes. Another difference between the present study and that by Chang *et al.* (2003) is the 48 h attachment period that the chondrocytes were given prior to adding the chemotactic agent to the lower well of the Boyden chambers (Chang *et al*., 2003),

whereas in the present study, there was no attachment period. Both of these differences may have allowed for phenotype changes to occur within the chondrocytes in the work by Chang *et al*. (2003), and therefore direct comparison between the present study and that of Chang *et al.* (2003) may not be entirely appropriate.

In terms of PDGF-bb, there is agreement between results from the present study and results from a previous study by Mishima and Lotz (2008). Mishima and Lotz (2008) found that 5 ng/mL PDGF-bb did not elicit significant chondrocyte chemotaxis relative to control groups, whereas 50 ng/mL PDGF-bb did elicit significant chondrocyte chemotaxis relative to control. In fact, 50 ng/mL PDGF-bb resulted in approximately 60% more chemotaxis relative to controls, which is comparable to the present study, in which 50 ng/mL PDGF-bb resulted in approximately 45% more chemotaxis relative to controls. It is interesting to note that the relative differences between the experimental groups and control groups in both the present study and that by Mishima and Lotz (2008) were consistent, even though the types of articular chondrocytes were different, as Mishima and Lotz (2008) used human chondrocytes. Other studies have investigated the potential of PDGF-bb to induce chondrocyte chemotaxis (Fujita *et al.*, 2004; Fukuyama *et al.*, 2004), but these studies used an immortalized chondrocyte line (MC3T3-E1), with results that may not be comparable to the present study, as the phenotype expression between primary chondrocyte and MC3T3-E1 cells is likely different. While the exact numerical differences in chemotaxis between experimental groups and control groups were not stated, it could be estimated that 100 ng/mL PDGF-bb resulted in approximately

100 times more chemotaxis relative to controls based on the figures presented by Fujita *et al*. (2004) and Fukuyama *et al*. (2004).

In terms of bFGF, the results from previous studies do not appear to be in agreement with each other, nor the present study. Maniwa *et al.* (2001) found that bFGF at concentrations of 1 ng/mL and 10 ng/mL resulted in significantly more chondrocyte chemotaxis in immature rabbit articular chondrocytes, but not at concentrations of 0.01 ng/mL and 0.1 ng/mL. Both 1 ng/mL and 10 ng/mL bFGF resulted in approximately 50% more chemotaxis relative to controls, with 10 ng/mL bFGF resulting in slightly more chemotaxis than 1 ng/mL bFGF. Conversely, Hidaka *et al.* (2006) found that 5 ng/mL bFGF was able to induce chemotaxis in immature bovine articular chondrocytes relative to controls, whereas 1 ng/mL and 10 ng/mL bFGF did not induce significant chemotaxis. While it is possible that species effects could be responsible for the differences between these two studies, the trends are interesting. In the Maniwa *et al.* (2001) study, there was an increasing trend apparent where higher bFGF concentrations resulted in more chemotaxis. Contrarily, in the Hidaka *et al.* (2006) study, there is no apparent trend between bFGF concentration and chondrocyte chemotaxis. The differences between the work of Hidaka *et al.* (2006) and the present study include the coating of the modified Boyden chamber with type II collagen by Hidaka et al. (2006), which may have confounded their results. Additionally, Maniwa *et al.* (2001) and Hidaka *et al.* (2006) terminated their chemotaxis experiments after only 1 and 6 hours, respectively, which may make comparisons with the present study difficult, as the chemotaxis experiments in the present study were terminated after 24 hours. The difference in the length of time

allowed for each experiment likely affected the number of cells that were able to migrate to the underside of the Boyden chambers.

A curious finding in the present study involves the chemotactic response of the chondrocytes when IGF-I, bFGF, and PDGF-bb were delivered at concentrations of 25, 50, and 100 ng/mL, which induced a significant chemotactic response. The trends in the data suggest that there may be a specific concentration of each growth factor required to induce chondrocyte chemotaxis between 10 and 25 ng/mL, as no dose-response was observed. However, no other studies have suggested any such potential behaviour of chondrocytes, possibly because of the lack of information currently available regarding chondrocyte chemotaxis.

5.4 Chemotactic outgrowth of chondrocytes from native cartilage

Chondrocytes displayed an increase in outgrowth from bovine articular cartilage explants when treated with 25 ng/mL IGF-I relative to untreated controls, indicating that results from the modified Boyden chamber experiments (which represent a model system to study chemotaxis) may be able to be applied to chondrocytes contained within explant tissue. However, the number of chondrocytes outgrowing from the explant tissue was small, and such a small number of cells would likely be unable to induce significant cartilage healing, which was the goal of the present work. Regardless, the fact that the IGF-I treated groups displayed significantly more outgrowth than control groups was used to further the present work and develop a method by which more chondrocytes could be encouraged to outgrow (§3.5). This result inspired the decision to use

collagenase treatment as a means of increasing the number of chondrocytes able to outgrow from explant tissue.

In the control group in the present study, 11 days were required for chondrocyte outgrowth. However, in a previous study using human articular chondrocytes, the time required for outgrowth was approximately 1 month (Qiu *et al.*, 2000). While there are likely inherent differences in the physical structure between human and bovine cartilage, the difference in time to outgrowth could be explained by age-related effects. In the present study, calf articular chondrocytes were used, whereas Qiu *et al.* (2000) used chondrocytes obtained from patients undergoing knee or hip arthroplasty. While the average donor age was not given by Qiu *et al.* (2000), it is likely that the chondrocytes were isolated from adult patients. A previous study has found that immature bovine articular chondrocytes have greater chemotactic potential than adult bovine articular chondrocytes (Hidaka *et al.*, 2006). In this study by Hidaka *et al.* (2006), it was found that chemotaxis was approximately three times greater in immature chondrocytes relative to adult chondrocytes. The results of the present study are in agreement with the age-related differences reported by Hidaka *et al.* (2006), as the outgrowth time for the immature bovine articular chondrocytes was one third of the time required for human articular chondrocytes as found by Qiu *et al.* (2000).

5.5 Collagenase penetration into cartilage explant tissue

While collagenase has previously been studied as a means of promoting chondrocyte outgrowth from cartilage (Qiu *et al.*, 2000; Bos *et al.*, 2002; van de

Breevaart Bravenboer *et al.*, 2004; Janssen *et al.*, 2006), none of these studies looked at the extent to which collagenase penetrates into cartilage following treatment. Additionally, Bos *et al.* (2002) and van de Breevaart Bravenboer *et al.* (2004) used collagenase treatment times of up to 48 hours. However, a 48 hour treatment time, if translated to the clinic, would require multiple operations in order to repair a defect, which is undesirable for both patients and surgeons.

One important reason why the extent of collagenase penetration into the tissue should be examined in depth relates to the fact that over-treating cartilage may degrade more ECM than is necessary. Tissue samples treated for 48 hours will likely be somewhat degraded throughout the entire sample. The implications of degrading the ECM within the entire sample could be that the sample could then have lessened structural integrity, and the chondrocytes would need to repair the structural damage done by the collagenase. However, adjusting the extent of collagenase penetration into a sample by varying the treatment time and concentration allows for only selective destruction of collagen; collagen within the interior of a sample could remain structurally intact.

Two studies have looked at reduced collagenase treatment times (Qiu *et al.*, 2000; Janssen *et al.*, 2006). Qiu *et al.* (2000) used treatment times between 1 and 15 min, and found that all treatments resulted in significantly reduced times required for cell outgrowth from tissue, such that longer treatment times resulted in faster chondrocyte outgrowth. Janssen *et al.* (2006), in wanting to reduce collagenase treatment times to times that would be clinically feasible, found that treating samples for 1 hour had the same effect as treating samples for 48 hours in terms of restoring chondrocyte density at

the edge of a wound. However, Qiu *et al.* (2000) and Janssen *et al.* (2006) did not investigate the effect of their treatments on the ECM, but rather the effect of their ECM-degrading treatments on chondrocytes.

One interesting finding in the present study was that the extent of collagenase penetration was dependent on the method of cartilage harvest. The depth of penetration was dependent on the distance from the articular surface in groups harvested with a sharp biopsy punch, but not dependent on the distance from the articular surface in groups harvested with dulled biopsy punches. A possible explanation for this phenomenon is that as a sharp object is passed through the tissue, it may leave the adjacent ECM intact, whereas a dull object that is passed through the tissue may significantly disrupt the ECM within the cartilage, which could disrupt the depth-dependent structure of the tissue. If the depth-dependent structure is disrupted, then the water content within the tissue may also be affected. An increase in water content in the deeper zones of the tissue could affect collagenase transport into the tissue in the deeper zones. Another possibility is that if the collagen network is disrupted, it may be more susceptible to collagenase breakdown due to a less structured formation. Regardless of explanation, the phenomenon exists, and actually gives support to the potential of treating cartilage with collagenase following osteochondral harvest. It is important to encourage chemotaxis of those chondrocytes that are able to generate new tissue once they reach the periphery of the graft. Previous research has demonstrated that chondrocytes within the superficial region of cartilage are unable to generate significant amounts of ECM at the tissue periphery (Bos *et al.*, 2008).

In contrast, chondrocytes from the deep region of cartilage are able to generate a significant amount of new ECM (Bos *et al.*, 2008).

It is important to note that previous studies did not account for depth dependence of collagenase penetration based on harvesting method. One previous study found that cartilage healing could be improved following collagenase treatment when cartilage is harvested with a biopsy punch (Janssen *et al.*, 2006). The images presented by Janssen *et al.* (2006) actually illustrated the same depth-dependent collagenase penetration into the tissue that was seen in the present study for a biopsy punch; however, the authors may not have recognized the phenomena, or they may have thought that it was insignificant. Although that previous study found that cartilage healing could be improved by collagenase treatment, there was evidence that the ECM surrounding the deep zone chondrocytes was not significantly disrupted. This may have prevented the deep zone chondrocytes from contributing to the healing response that was found. This observation is encouraging in that it is possible that healing may have been further improved had a uniform depth-dependent collagenase penetration been observed, as the deep zone chondrocytes may have been able to contribute more to the healing.

The data obtained in the present study regarding collagenase penetration into cartilage was used only to determine an appropriate treatment concentration and time for a proceeding experiment. While a significant amount of data was collected, more data would be required in order to develop a functional model that allows for prediction of the depth of collagenase penetration into cartilage based on treatment time and concentration. Such a model may not only be useful in its application to the current method of treating

cartilage with collagenase as a means of improving cartilage healing, but may also be important in other work examining the effects of the matrix metalloproteinases (ECM-degrading enzymes) on their ability to penetrate into and degrade cartilage.

5.6 Collagenase digestion and chondrocyte outgrowth from native cartilage

Collagenase digestion led to significantly increased chondrocyte outgrowth from native cartilage. This finding agrees with previous work directly examining chondrocyte outgrowth from cartilage (Qiu *et al.*, 2000), as well as other work that found collagenase treatment resulted in higher chondrocyte density near the wound edge of samples treated with collagenase (Bos *et al.*, 2002, Janssen *et al.*, 2006). However, because the collagenase source between the present study and those mentioned above differs, direct comparisons between collagenase treatments are difficult to make. The collagenase supplier for the present study was Roche, whereas the collagenase supplier for each of the above-mentioned studies as Sigma-Aldrich. The standard for measuring collagenase activity between these two suppliers is different, with Roche stating activity in Wünsch units, and Sigma-Aldrich stating activity in Mandl units, and unfortunately only an approximate conversion exists, where 0.15 U/mg in Wunsch units is approximately equal to 250 U/mg in Mandl units. A table with standardized collagenase concentrations between each of the above-mentioned studies and the present study is given in Table 5-1.

Table 5-1. Comparison of collagenase treatment concentrations used between the present study and various other studies.

Author	Collagenase concentration (Mandl units/mL)	Treatment time
Present study	~250	10 min
Qiu *et al.* (2000)	380	1 – 15 min
Bos *et al.* (2002)	30	48 h
Janssen *et al.* (2006)	10	48 h

While the collagenase treatment concentrations are different between the present study and Qiu *et al.* (2000), both studies found that short duration collagenase treatment can result in decreased time required for chondrocyte outgrowth. One apparent difference between the present study and that of Qiu *et al.* (2000) is that although the present study used a lower collagenase concentration, outgrowth was achieved after one day in culture, whereas Qiu *et al.* (2000) found that the time required for outgrowth following 15 min collagenase treatment required an average of 2.2 days for outgrowth. While this difference may be inherent to the collagenase treatment times and concentrations used, it may also be explained by the fact that in the present study, young articular cartilage was used, whereas Qiu *et al.* (2000) likely used adult articular cartilage. As mentioned previously, there are age-related differences in chemotactic potential of bovine articular chondrocytes (Hidaka *et al.*, 2006), and this difference could be extended to include the general migration potential of chondrocytes as well. Such a phenomenon could explain the fact that the bovine articular chondrocytes in the present study were able to migrate to the tissue periphery in less time than adult human articular chondrocytes, despite the fact

that the bovine cartilage was treated with a lower concentration of collagenase for a shorter period of time.

When collagenase digestion was coupled with IGF-I supplementation, there were significant increases in chondrocyte outgrowth compared to collagenase-only treated samples. This finding agrees with the previous work presented in the present study that IGF-I promotes chondrocyte chemotaxis in both a modified Boyden chamber and in native bovine articular cartilage. As it has been mentioned previously, collagenase treatment alone allows for increased chondrocyte migration to the tissue periphery, and adding a chemotactic agent following collagenase treatment presents a method by which increased numbers of chondrocytes may be encouraged to reach the periphery of a cartilage explant. This result may indicate that collagenase treatment coupled with IGF-I supplementation could lead to increased cellularity at the periphery of a cartilage wound or cartilaginous portion of an osteochondral graft, and may result in an improved healing response and possible integration with adjacent cartilage.

5.7 Overcoming the zone of chondrocyte death

While a previous study found that collagenase treatment alone allowed for healing of a cartilage wound (Janssen *et al.*, 2006), no such healing was achieved in the present study. However, the results of the present study are positive in that they demonstrated that chondrocytes are able to repopulate the ZCD. In the present study, there was no cartilage healing due to an absence of ECM bridging two pieces of cartilage. One possible reason why the present study did not result in healing is because a ZCD was

generated in the present study, whereas no ZCD was generated in the earlier study. Without a ZCD, the distance that chondrocytes would need to migrate in order to reach the tissue periphery would be significantly reduced. As such, the time required for chondrocyte migration to the tissue periphery would also be reduced. Additionally, the previous study used an *in vivo* healing model, where explant tissue was implanted in the backs of nude mice, giving a significantly different culturing environment than that in the present study. It is possible that increasing the culture period in the present study could have resulted in greater repopulation of the ZCD and eventual outgrowth from explant tissue. This could have then resulted in tissue formation at the periphery, and eventual tissue formation between the two cartilage pieces. It is important to note that it has previously been suggested that an *in vitro* culture system may not provide an appropriate environment for tissue growth (Obradovic *et al.*, 2001), which may have been the root cause of the lack of tissue formation between the cartilage pieces. Although no cartilage healing was observed in the present study, it is an encouraging finding that chondrocytes were able to migrate into the ZCD region of the tissue. This result has several implications, to be discussed below.

One implication of the present study is that the viability of the cartilaginous portion of osteochondral grafts could be significantly increased. As mentioned previously, the cartilaginous portion of a 4.5 mm osteochondral graft with a 400 um ZCD would have been only around 70% viable tissue. If chondrocyte migration into the ZCD could be maintained beyond four weeks, as per the present work, it may be possible to restore the tissue viability to 100%. This could allow for turnover of the tissue within the

ZCD region, allowing the tissue to remain healthy. It has been noted that the cartilaginous portion of the grafts can fissure over time, possibly to lack of viable tissue in the graft (Hurtig *et al.*, 2001). Increasing graft viability by allowing chondrocytes to migrate into the ZCD region could eliminate this problem.

Another implication of the present work is the possibility that if chondrocyte migration into the ZCD continued beyond four weeks, it may be possible to encourage the chondrocytes to migrate to the periphery of the tissue, and possibly beyond. Chondrocytes at the periphery of the tissue have the potential to generate tissue that allows bridging between adjacent pieces of cartilage. Additionally, chondrocytes at the periphery of the tissue could generate high quality hyaline cartilage within the void regions between osteochondral grafts following mosaic arthroplasty. Presently, fibrocartilage develops between the grafts (Hangody *et al.*, 1998), which ultimately wears away. However, as the fibrocartilage wears away, the result could be that there are greater forces exerted on the grafts, which could potentially explain why graft failures have been noted in the past (Hunziker, 2002). If fibrocartilage could be replaced by hyaline cartilage, such an issue could potentially be avoided. The results of the present study indicate that there is a potential that chondrocytes from within the cartilaginous portions of the osteochondral grafts could generate tissue allowing for tissue regeneration within the void spaces between grafts. Because any such tissue regeneration within the void region could contain chondrocytes that have migrated from surrounding cartilage, there is a possibility that if blood can be excluded from the void regions between the grafts, only primary, undifferentiated chondrocytes would be generating tissue. The region between

grafts sees an influx of blood as a result from the surgical procedure, which brings with it MSCs, and the MSCs generate fibrocartilage to fill the space (Minas and Nehrer, 1997, Hangody *et al.*, 1998). However, even if MSCs could be encouraged to differentiate toward a chondrogenic phenotype, there remains the question of whether they would be able to generate tissue that resembles native cartilage. If primary chondrocytes are generating tissue between grafts, there is the potential that they will generate hyaline-like cartilage.

5.8 Chondrocyte chemotaxis through methacrylated glycol chitosan

Three-dimensional chondrocyte chemotaxis through mGC could not be achieved in the present study. Typical chemotaxis assays are two dimensional in nature, and do not account for the significant cell motility that is required in order to achieve chemotaxis *in situ*, where there are barriers preventing cell movement through tissue. The hydrogel used in the present study, mGC, had an important property that could have been combined with findings in the present study. That is, that mGC could be photocrosslinked *in situ* between grafts following mosaic arthroplasty. The use of a photocrosslinkable hydrogel was an attractive approach, as the spaces between grafts following mosaic arthroplasty vary between patient, and the gel could be made to fit any dimension required. However, as chondrocytes seeded on top of the gel could not survive, the assay failed to demonstrate chondrocyte chemotaxis through a three-dimensional network. The approach of the current study differs from the approach in a previous study (Amsden *et al.*, 2007) in that the crosslinking time and intensity was different, the cell source used was

different, a different physical setup was used, and a different type of media was used. With respect to the type of media, the ITS used in the present study may not have allowed for sufficient ECM production of the cells immediately after seeding, and ECM production is required in order for cells to attach to a hydrogel and survive. Thus, no definitive comparisons can be made between the present study and that done by Amsden *et al*. (2007).

While chondrocyte migration into mGC could not be achieved using the ITS media + IGF, it does not completely rule out the possibility of using mGC in practice to fill the spaces between grafts. While chondrocytes seeded on the surface of an mGC hydrogel did not maintain viability after a short period of time under the culture conditions used, chondrocytes seeded on the surface of mGC have been shown to migrate into the hydrogel and retain high viability after one week in culture (Amsden *et al.*, 2007). Thus, further work is required in order to determine the suitability of mGC as a material that may be used to fill the spaces between osteochondral grafts following mosaic arthroplasty.

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

Mosaic arthroplasty is a cartilage resurfacing technique whereby osteochondral grafts are harvested from non-weight-bearing areas of the knee and implanted into cartilage defect sites. While mosaic arthroplasty gives good healing outcomes, problems associated with the procedure include poor integration of the cartilaginous portions of grafts and fibrous tissue formation between grafts. Such problems are likely due to the zone of chondrocyte death that forms as a result of graft harvesting, which can extend up to 400 μm into the tissue. Chondrocyte migration through this zone to the wound edge is inhibited by dense ECM, but it has been previously suggested that increasing the number of chondrocytes at edge of a cartilage wound could allow for improved cartilage healing and integration (Janssen, 2006, van de Breevaart Bravenboer, 2004).

In the present study, the chemotactic response of bovine articular chondrocytes to IGF-I has been demonstrated in four methodologically different experiments (§4.3, §4.4, §4.6, §4.7). Additionally, the chemotactic response of bovine articular chondrocytes to bFGF and PDGF-bb has been demonstrated in two methodologically different experiments (§4.3, §4.7). At present, there have been few numbers of studies investigating chondrocyte chemotaxis, and of those studies, very few have replicated the findings of others. The results of the present study add to the available literature on

chondrocyte chemotaxis, and may be used by other researchers in the future to guide further work on the subject.

Bovine articular chondrocyte outgrowth from explant tissue has also been shown to increase following treatment with collagenase, which supports existing literature. While no significant attempts have been made to translate collagenase use from the laboratory to the clinic, the results of the present study can at least lend support to the collagenase work that has been done thus far. Furthermore, the majority of the work done on the use of collagenase as a treatment for cartilage injuries has been undertaken by a single research group in the Netherlands. As the results of the present work are in agreement with this research group, it may increase the legitimacy of exploring the use of collagenase treatment as a means of promoting cartilage healing and integration.

The major finding in the present study was that treating cartilage with collagenase for a short period of time caused sufficient breakdown of cartilage ECM to allow IGF-1 supplementation to generate a chemotactic response of chondrocytes such that they were able to repopulate a ZCD. This result could enable chondrocyte migration to the periphery of a cartilage wound or cartilaginous portion of an osteochondral graft, potentially allowing for integration and healing between adjacent pieces of cartilage. However, in order to definitively conclude that the method developed in the present study can allow for improved cartilage healing, more work needs to be done on the topic, and several questions must be addressed before the work can be translated from the laboratory to the clinic.

6.2 Recommendations

One question that must be resolved is whether the method developed in the present study can actually allow for improved cartilage healing. As mentioned previously, an *in vitro* culture system may not be appropriate for generating tissue. As such, it may appropriate to repeat the healing study in the present work (§3.6) in an *in vivo* system, where cartilage healing may be more likely to occur.

It has been recognized that surgeons may be hesitant to treat osteochondral grafts with collagenase for fear that the collagenase could compromise the integrity of the transplanted cartilage following mosaic arthroplasty. In the present study, cartilage was bathed in collagenase and the articular surface was degraded as a result. In order to prevent degradation of the articular surface, investigations should be made into how to preserve the articulating surface during collagenase treatment. Furthermore, the long-term effects of collagenase treatment should be investigated to determine if the degraded regions are remodeled by the chondrocytes. It may also be worth while to investigate how collagenase treatment affects the integrity of the cartilaginous portion of the osteochondral graft through mechanical testing. Finally, because different surgeons use different osteochondral harvesting instruments, a recommended collagenase treatment may be dependant on the instrument used, as each instrument may induce a different ZCD. However, more work needs to be done in order to develop a functional model by which collagenase treatment time and concentration can be used to estimate the extent of collagenase penetration into the side of cartilage tissue.

Additionally, more work must be conducted in order to determine if more chondrocyte migration into the ZCD can be achieved if a longer culture time is used, or if an *in vivo* system is used. If more chondrocyte migration into the ZCD can be achieved over time, this could make the make the method developed in the present study more attractive. Furthermore, additional work is required in order to determine if chondrocyte migration can be achieved beyond the ZCD to the periphery of the tissue, and potentially into the void spaces between osteochondral grafts.

More work must be carried out on investigating the ability of chondrocytes to migrate into mGC. While the present study does not support the idea that chondrocytes can migrate into mGC, there is another body of work that does support the idea (Amsden *et al.*, 2007). The ultimate goal of the present work is to allow chondrocyte migration into a material between osteochondral grafts following mosaic arthroplasty so that new tissue could be generated within this material. Therefore, it may be worthwhile to determine if chondrocytes are able to migrate directly from cartilage into mGC. If mGC is not an appropriate material for filling the space between osteochondral grafts, other materials must be investigated. However, other materials should also be able to be photocrosslinked *in situ*, as the shape of the space between osteochondral grafts varies from patient to patient, and thus, the shape of the material must be able to adapt.

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Appendices

Appendix A: Depth of collagenase penetration

The depth of penetration for various collagenase concentrations and time are given below using the OATS osteochondral system (Figure A.1) and a sharp biopsy punch (Figure A.2).

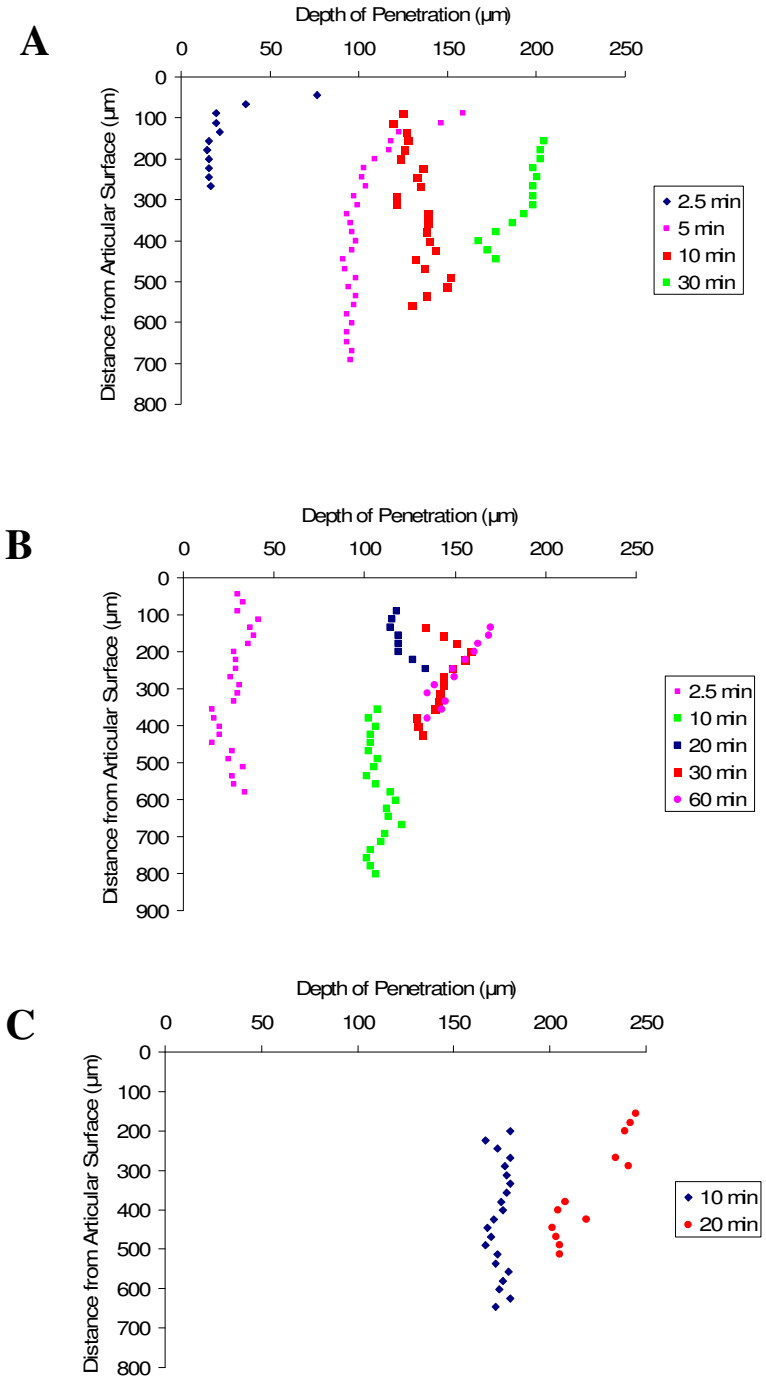

Figure A.1. Depth of collagenase penetration into cartilage tissue harvested using the OATS osteochondral system and treated for various amounts of time with collagenase at a concentration of 0.15% (A), 0.3% (B), or 0.6% (C) collagenase.

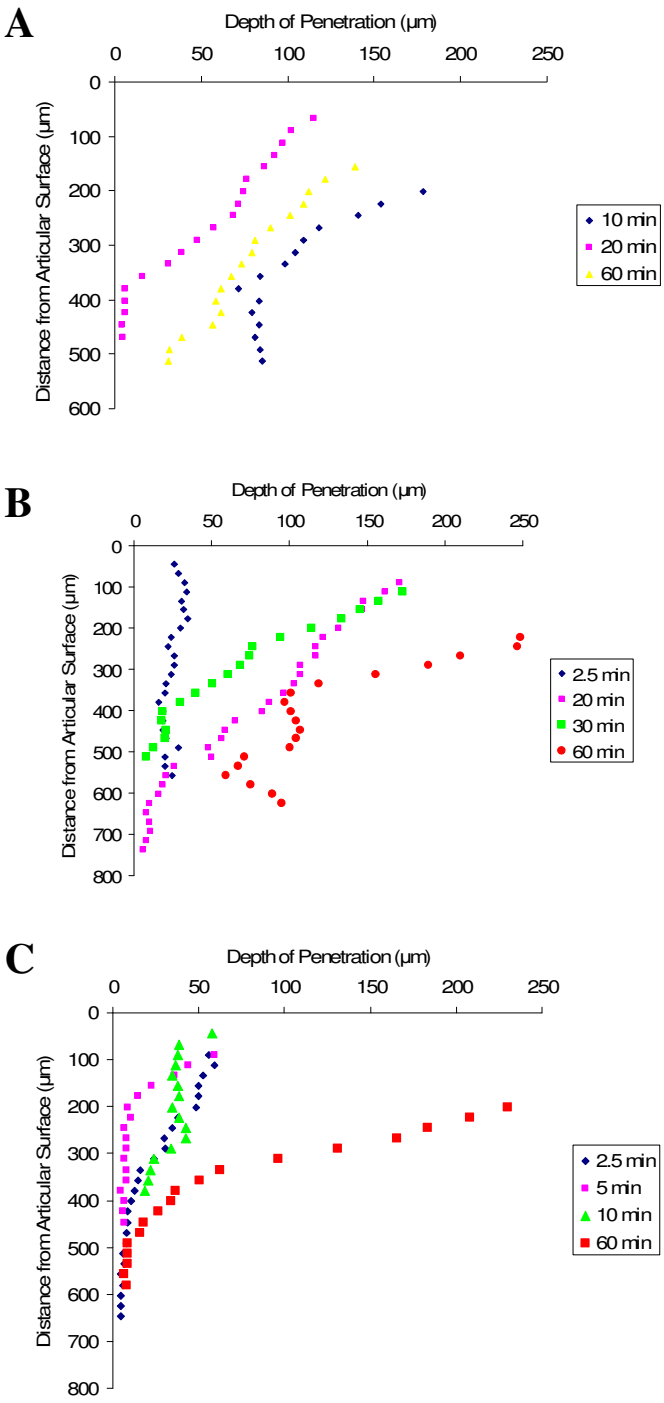

Figure A.2. Depth of collagenase penetration into cartilage tissue harvested using a sharp biopsy punch and treated for various amounts of time with collagenase at a concentration of 0.15% (A), 0.3% (B), or 0.6% (C) collagenase.

Appendix B: Pilot study examining repopulation of the zone of chondrocyte death

A pilot study was conducted to determine the feasibility of treating cartilage with collagenase and supplementing with a chemotactic agent as a means of reducing the ZCD. A description of the study is given below.

Bovine articular cartilage explants were harvested and a ZCD was induced using the OATS osteochondral harvesting tool. Explants were either treated for 10 minutes with 0.15% collagenase or left untreated. After enzymatic disruption of the ECM, samples were cultured in serum-free media supplemented with or without IGF-1 (25 ng/mL). Cultures were harvested weekly (at 7, 15, 21 days) and stained with calcein AM and ethidium homodimer to assess cell viability. The ZCD was measured using confocal microscopy and statistically compared amongst the different treatment groups. Results indicated that there was a significant effect of the various culture conditions on the ZCD ($p < 0.001$). Collagenase treatment reduced the ZCD by approximately 40% compared to control (*collagenase:* 140 ± 17 μm; *control:* 241 ± 21 μm). Although IGF-1 supplementation alone had no discernable effect on the ZCD, IGF-1 supplementation after pre-treatment with collagenase significantly reduced the ZCD by 80% compared to control (*IGF-1:* 255 ± 13 μm; *collagenase + IGF-1:* 48 ± 13 μm). In addition, there appeared to be a linear trend in the collagenase + IGF group whereby the ZCD decreased over time (*Day 7*: 65 ± 18 μm; *Day 21*: 34 ± 9 μm). Thus, treating osteochondral grafts with collagenase and IGF-1 induces chondrocyte repopulation of the ZCD generated by osteochondral graft harvesting.

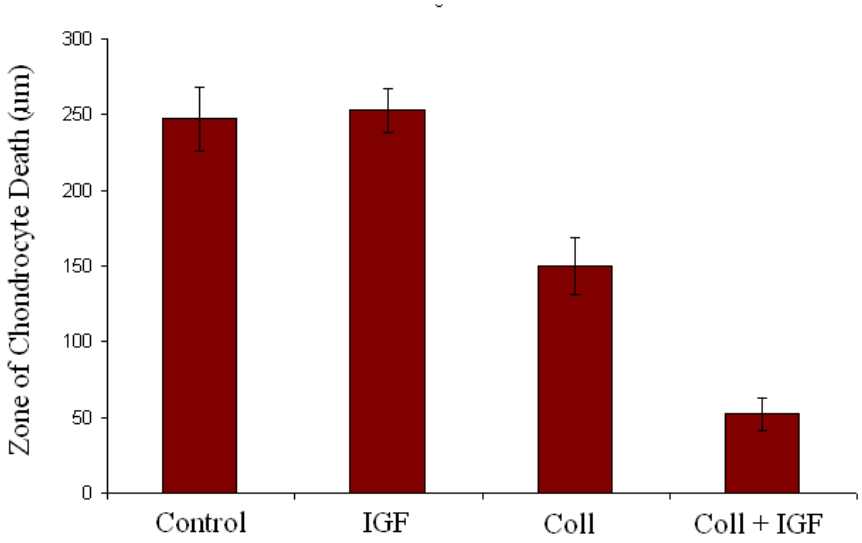

Figure B.1. The effect of explant pretreatment and culture conditions on the zone of chondrocyte death.

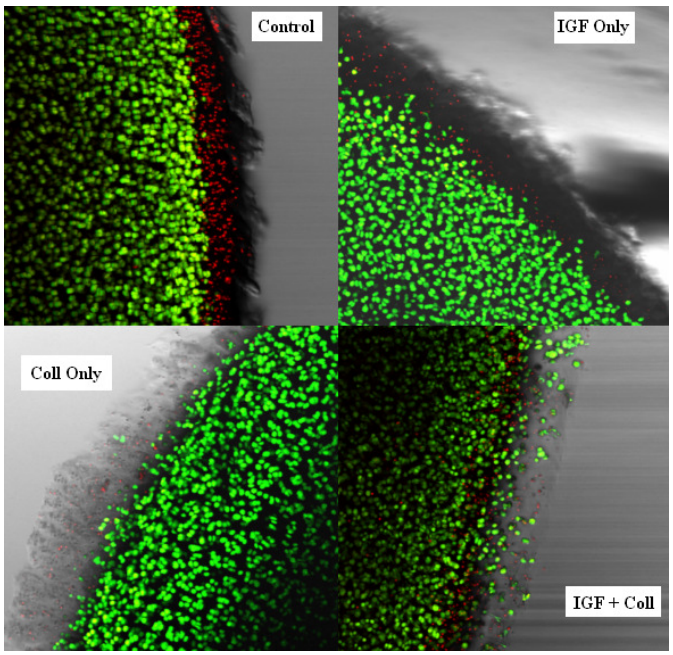

Figure B.2. The effect of explant pretreatment and culture conditions on the zone of chondrocyte death as viewed by confocal microscope.