PREVENTION AND TREATMENT OF POST-TRAUMATIC OSTEOARTHRITIS

by

Miri Park

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomechanics and Movement Science

Winter 2016

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ACKNOWLEDGMENTS

I would first like to thank my advisor, Dr. Lucas X. Lu for his excellent guidance and support he provided. He is the most motivational, hardworking and enthusiastic scientist with full of passion in orthopaedic research. With his busy schedule, he always closely worked with us and checked on us with every step of experiments. It really was a great joy and honor for me to learn and contribute to his lab.

I am also very grateful for my thesis committee, Drs. Liyun Wang, Catherine Kirn-Safran and Lin Han. These professors have been always available and give best intellectual advice for my study. I am very grateful for their time, interest in my study, and helpful comments.

The work could not be completed with my dear fellow labmates, especially Yilu Zhou. Yilu and I have been working closely together from the first year in the lab. It has been great working together with him for the past 4 years who has been always reliable source of scientific help. I would also like to acknowledge Monideepa Chatterjee and Anna Sung for being the best assistant for me. They are very hardworking and talented undergraduate students who provided a lot of pipetting and editing help whenever I needed. I am also grateful for my labmates, Brandon Zimmerman and Enoch Cheung for their contribution to my work. In addition, Mengxi Lv, Xingyu Chen and Jie Ma helped me in all aspects and helped my dissertation possible. I would also like to acknowledge my fellow scientist friends, Woojin Han, Wen Li, Aidan Zerdoum, Zhixiang Tong, Padma Pradeepa, and Matthew Rehman, for the academic and scientific help. I have learned many valuable experimental technics from them and very grateful to have met and known them at UD.

Finally, I would like to acknowledge my friends and family who supported me during my time here. My dear friends, Sagaw (Fai) Prateepchinda, Minji Choi, Yehmi Kim, Jeong-min Lee, and Henry Wang, kept me stay sane through this difficult time. Thanks to their friendship and encouragements. Their care and support helped me to overcome and stay focused. Most importantly, I would like to thank my family for their constant love and support. Their unconditional love has been the most basic source of my life energy. Thank you everyone!

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ABSTRACT

Osteoarthritis is the leading cause of disability among adults in the United States. Traumatic damage to joints, such as meniscal or ligament tears, can induce cartilage degeneration and lead to post-traumatic osteoarthritis (PTOA). Treatments of PTOA are currently similar to those for idiopathic OA. When small cartilage lesions are diagnosed, microfracture surgery often serves as the first line option for repair. However, clinical practice and animal studies have yielded variable and unpredictable healing results after microfracture surgery. In the first half of this thesis, a novel *in vitro* cartilage-bone explant co-culture system was developed which can simulate the microfracture procedure and post-surgery rehabilitation protocol. This *in vitro* platform avoids the high financial cost and complexities of *in vivo* systems, and also enables the longitudinal measurement of the biomechanical and biochemical properties of the newly repaired tissue. Using this system, we found that the availability of TGF-β3 to the bone marrow clot can significantly improve the growth of new cartilage at the lesion site. Moreover, a two-week delayed mechanical stimulation on the cartilage-bone explant generated better outcome than immediate loading after microfracture procedure. This finding implies that a delayed weight bearing on the knee joint after microfracture surgery may improve the long-term outcome.

Few preventive treatments exist for PTOA, although osteoarthritis could often be predicted after joint injuries. It has been found that bisphosphonates (BP), an FDA-approved drug to treat bone loss, could suppress the development of osteoarthritis after

various joint injuries in animal studies. The second half of this thesis aims to test a novel hypothesis that the chondro-protective function of BP is related to its direct effect on the chondrocytes. Using *in vitro* culture system, we found that BP can promote the beneficial effects of mechanical stimulation on cartilage explants without the presence of bone. The expression of anabolic genes in chondrocytes was promoted, while the catabolic genes downregulated. This regulation of metabolic activities of chondrocytes by BP is related to the spontaneous intracellular calcium signaling, the fundamental second messenger for cells. Moreover, the effects of BP on chondrocytes are dependent on the inhibition of mevalonate pathway. Therefore, we also investigate the effects of statins, another family of drug targeting at mevalonate pathway, on the long-term culture of cartilage explants. Statins showed a biphasic effects on the chondrocytes. In short term, stains promoted the expression of anabolic genes in chondrocytes. After long term culture, in contrast, statins reduced the mechanical integrity of cartilage. Therefore, the FDA-approved drugs that inhibit the mevalonate pathway have the potential to prevent or delay the PTOA progression. This study sheds new light on the search for new classes of drugs to inhibit trauma-induced OA.

Chapter 1

INTRODUCTION

1.1 Articular Cartilage

Articular cartilage, which is a soft connective tissue covering the articulating surface of long bones within diarthrodial joints, provides the joints with essential biomechanical functions. The tissue is composed of 80% water and structural macromolecules such as collagen and proteoglycans (Cohen, Foster et al. 1998). There is only one cellular type residing in articular cartilage, the chondrocyte, which is surrounded by extracellular mat**r**ix (ECM). Type II collagen is the most abundant protein linked together within the ECM (Jasin 1995). Proteoglycan is a large aggregating macromolecule which contains aggrecans linked with hyaluronan (HA), link proteins (Seror, Merkher et al. 2011), and a plethora of lateral glycosaminoglycans (GAGs), which have fixed negative charge. These fixed charges on GAG chains generate the osmotic pressure inside the tissue which can sustain compressive loadings and retain water in the tissue.

There are four different zones inside the articular cartilage: the superficial zone, the middle zone, the deep zone, and the calcified zone (Figure 1.1), reflecting different ultrastructure of ECM at different depth. The superficial zone, which composes 10-20% of the cartilage thickness, protects the other deeper layers from shear stresses. The collagen fibers of this superficial zone are packed tightly and aligned parallel to the articular surface. The middle zone provides an anatomic and functional bridge between the superficial and deep zones. It comprises of 40 to 60% of the total cartilage volume and contains proteoglycans and thick collagen fibrils. The deep zone provides the greatest resistance to compressive forces, and the collagen fibrils are arranged perpendicular to the surface. The calcified layer plays an integral role in securing the cartilage to bone, by anchoring the collagen fibrils in the deep zone to the subchondral bone.

In terms of the mechanical properties, articular cartilage is a typical viscoelastic material. Besides the intrinsic viscoelastic properties of the solid matrix (collagen-proteoglycan network), the frictional drag between fluid flow and solid matrix contributes significantly to the viscous behavior of cartilage due to extremely small pore size of ECM. This flow-dependent viscoelastic character can be precisely described by a poroelastic or biphasic theory (Mow, Kuei et al. 1980, Mow, Holmes et al. 1984, Simon, Coats et al. 1984). In daily activities, the fluid pressure may sustain most of the compressive loading (> 95%), and transferred the principal stress on the collagen-proteoglycan network into tension, which is more preferable for the solid matrix to sustain. This mechanism protects the cartilage from trauma damage and is critical for the maintenance of joint health.

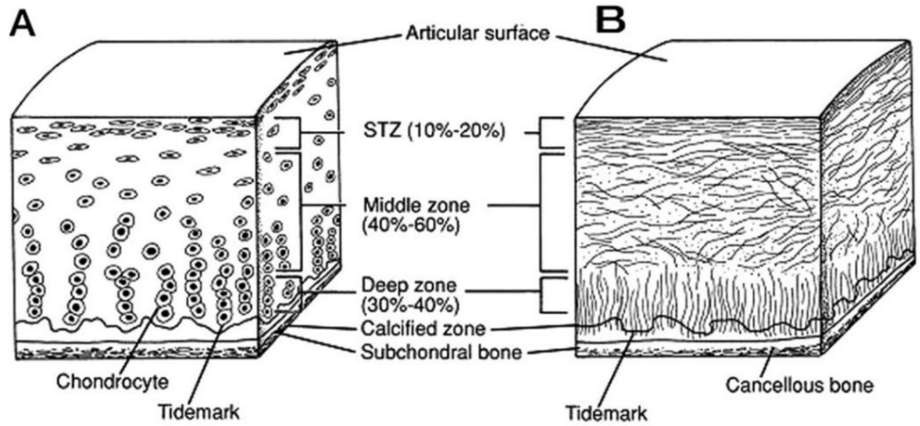

Figure 1.1 Schematic and cross-sectional diagram of healthy articular cartilage. A. Zonal organization of articular cartilage. B. Collagen fiber architecture. (Buckwalter, Mow et al. 1994).

The articular cartilage have high tensile strength, which originates from network of collagen fibers in ECM (Cohen, Foster et al. 1998). These collagen fibers, produced by chondrocytes, are organized in a highly ordered manner to provide the tissue with superior capabilities to sustain tensile load. Proteoglycans and non-collagenous proteins bind to the collagenous network and help to stabilize the matrix (Bank, Bayliss et al. 1998). The matrix protects the cells from injury during the normal use of the joint, and determines the types and concentrations of molecules that reach the cells and helps to maintain the chondrocyte phenotype.

Being surrounded by the ECM, chondrocytes senses the mechanical and biochemical changes in ECM via various cell surface receptors and responds through signaling events (Cohen, Foster et al. 1998). Therefore, the cell behaviors, such as polarity, migration, differentiation, proliferation and survival, are largely regulated by the stiffness, ultrastructure and composition of ECM. As the cartilage is avascular, chondrocytes only receive nutrients from the diffusion of joint synovial fluid through the pores of ECM (Buckwalter, Mankin et al. 2005).

1.2 Post-Traumatic Osteoarthritis (PTOA)

Osteoarthritis (OA), which is generally characterized as a slowly progressive degeneration of articular cartilage, is a disease that can disable the whole joint. As the joint surface wears away, the core of the bone becomes exposed. The bone rubs against the other bone or nearby soft tissues which causes significant pain and joint swelling (Figure 1.2). Note that articular cartilage is deficient of blood vessels, lymphatics, and nerves. Therefore, cartilage has limited capacity of intrinsic healing and regeneration, and the diagnosis of cartilage degeneration at early stage remains challenging in practice. OA is the leading cause of disability among Americans and is responsible for over 600,000 joint replacements each year (Gill, Moezzi et al. , 2012). Approximately 50% of patients develop OA due to traumatic joint injuries.

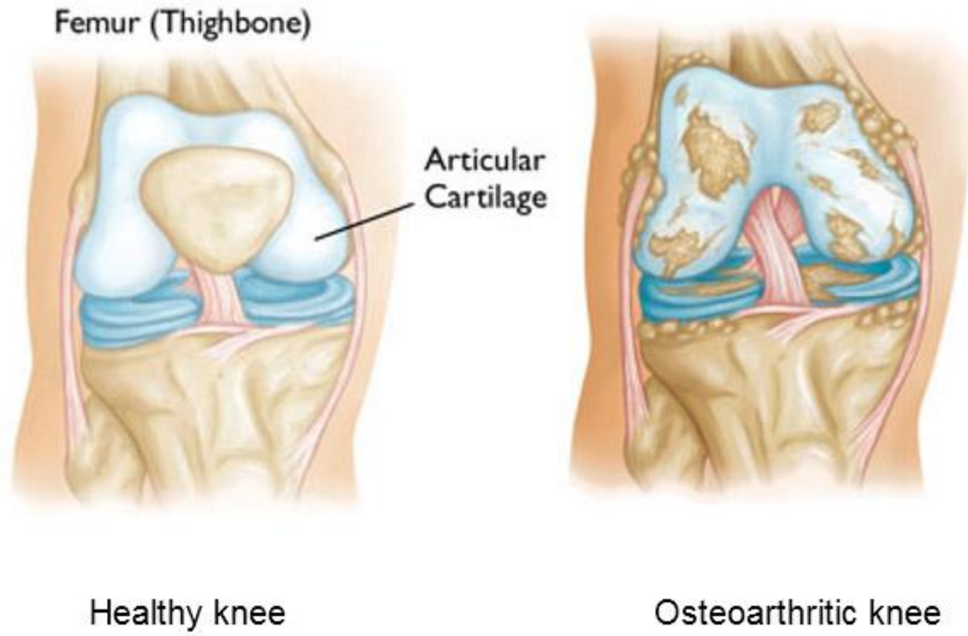

Figure 1.2 Healthy knee on the left and osteoarthritic knee on the right side. OA is usually a slowly progressive degenerative disease in which the joint cartilage gradually wears away (Adapted from the American Academy of Orthopaedic Surgeons website).

Post-traumatic osteoarthritis (PTOA) is an accelerated development of OA due to joint trauma, e.g., meniscal tear and anterior cruciate ligament (ACL) rupture. Approximately 12% of adults with OA in the United States are caused by joint trauma with an annual cost of $3 billion (Brown, Johnston et al. 2006). PTOA often occurs in young people with excessive physical activities (Buckwalter 2003, Anderson, Chubinskaya et al. 2011). Athletes are more likely to experience an ACL injury than regular people which can lead to high medical expense and discontinuation of their athletic activity. It has been reported that knee injuries account for 60% of all-sports related surgeries, while 50% of those knee injuries are ACL related (Majewski, Susanne et al. 2006).

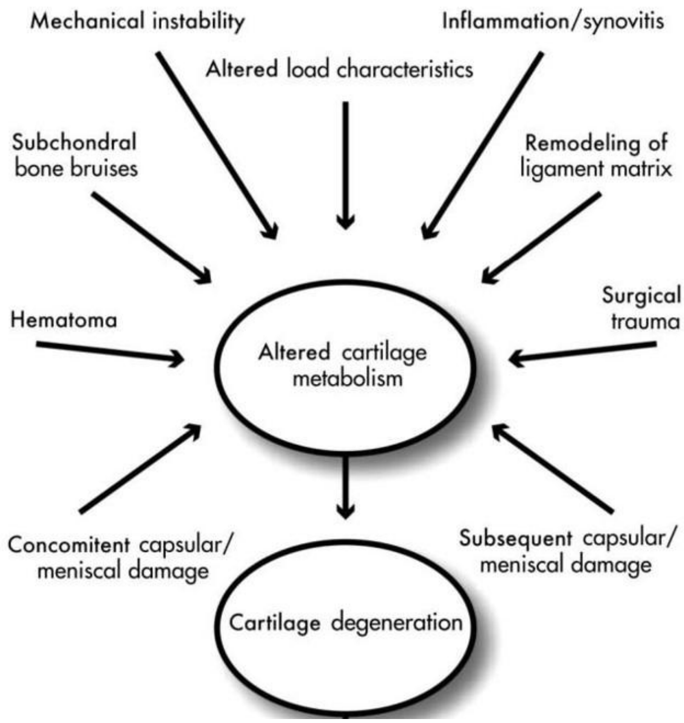

Figure 1.3 Factors that contribute progression of osteoarthritis following ACL injury. Factors such as mechanical instability may alter cartilage metabolism, which in turn causes cartilage breakdown, leading to arthritis (Fleming, Hulstyn et al. 2005).

Etiology of PTOA is not fully understood and most likely multifactorial (Figure 1.3). An ACL injury alters the kinematics of the knee, placing the ACL-deficient patient at risk for early osteoarthritis. Joint trauma affects all joint tissues to some degree but the damage to articular cartilage appears to be the most significant. Collagen and proteoglycan synthesis is suppressed and matrix-degrading enzymes and inflammatory mediators are over-expressed (DiMicco, Patwari et al. 2004, Lee, Fitzgerald et al. 2005). This altered metabolism of chondrocyte and apoptosis may play a critical role in the development of PTOA (Szczodry, Coyle et al. 2009).

It is appealing to envision a therapy that could prevent, delay, or effectively manage knee OA in the young patient with traumatic joint injuries. The traditional first-line therapeutic options to the post-traumatic knee OA consists of non-pharmacological treatments such as weight loss, foot wedges, bracing and physical therapy. Pharmacological treatments include analgesics, nonsteroidal anti-inflammatory drugs, opioids, hyaluronic acid or corticosteroid injections. However, none of these techniques have been shown to alter the disease progression (Stiebel, Miller et al. 2014). As a result, many young OA patients will eventually be faced with the decision to either undergo surgical intervention or to continue with ineffective conservative therapies. Patients under 40 years of age will almost undoubtedly outlive the implant, necessitating riskier second surgery.

1.3 Cartilage Repair Techniques

Traditional technique for articular cartilage repair, e. g. debridement, attempt to reduce pain and to restore functionality of cartilage tissue. The success of these techniques rely on the similarity of the repair tissue to native articular cartilage in terms of the biochemical composition and mechanical properties (Newman 1998), yet the repaired tissue barely satisfies. Current clinical treatment options include osteochondral autograft transplantation (Gill, Moezzi et al.), microfracture surgery, autologous chondrocyte implantation (ACI), and tissue engineering techniques using cells and biomaterials to replace damaged cartilage and bone (Beris, Lykissas et al. 2005, Behrens, Bitter et al. 2006, Kon, Verdonk et al. 2009). The surgery can be classified into three main categories: osteochondral transplantation (OAT or mosaicplasty), subchondral marrow stimulation (Pridie drilling or microfracture), and ACI (Table 1.1).

In OAT, cartilage is transferred from barely loaded areas of the joint and implanted in a mosaic-like pattern in debrided cartilage defects (Hangody, Feczko et al. 2001). OAT is ideal for patients with small areas of cartilage defect. Drawbacks of this technique is that there are limited available donor tissue and donor site could develop morbidity (Hangody, Vasarhelyi et al. 2008). Also practically, it is difficult to make the cartilage surface to be flat.

Autologous chondrocyte implantation (ACI) has been studied extensively in the last two decades. In a pioneer trial (Peterson, Minas et al. 2003), a cartilage biopsy is taken arthroscopically from a nonweight-bearing area of the joint. Autologous chondrocytes were harvested and expanded for 3 to 5 weeks. The open surgical procedure follows to implant the newly grown cells into the cartilage defect. ACI has the advantage of using the patient’s own cells, and can be performed arthroscopically and minimally invasive, yet it requires two separate procedures.

Unlike ACI, microfracture requires single-stage arthroscopic procedure which could be quick, low morbidity and cost-effective. Of the numerous techniques available today, microfracture has been chosen as the first line treatment option for its simple and safe procedure. One of the concerns with microfracture is that it produces fibrocartilage that is less durable and not as strong as the hyaline cartilage. Many tissue engineering approaches have been adopted to improve the quality of the newly generated tissue after microfracture surgery, which will be introduced later.

Table 1.1 Cartilage repair techniques (OAT, Microfracture and ACI) and their advantages, disadvantages and outcomes.

Techniques	Advantages	Disadvantages	Outcome	Reference
OAT	Simple single-stage arthroscopic procedure Uses patients' own tissue Maintain hyaline cartilage	Donor site morbidity Limited tissue size and supply surface even	86% of patients reported an increased level of joint functions Gold Standard	(Jakob, Franz et al. 2002, Marcacci, Kon et al. 2005)
Microfracture	Simple single-stage arthroscopic procedure Low morbidity Cost-effective	Produce fibrocartilage Difficulty in rehabilitation	80% of patients rated to have improved knee functions at 7 years postoperative	(Steadman, Briggs et al. 2003)
ACI	Potential for restoration of hyaline cartilage Treatment of larger lesions	Two stage procedures: time consuming, Expensive Laboratory support required: highly technique dependent Long recovery time	82% patients reported good to excellent clinical results after 2 years	(Peterson, Brittberg et al. 2002)

1.4 Significance of This Study

Currently, there are no therapeutic techniques available to prevent the progression of PTOA. Symptoms of cartilage degeneration often appear half to several years after the initial joint injury. Due to the challenge in the early detection of cartilage degeneration, PTOA usually presents as moderate-to-severe degeneration at the time of diagnosis. This makes the interventions of PTOA similar to those for late-stage primary OAs, such as microfracture, ACI, and passive physical therapy (Wilson, Michet et al. 1990). Ultimately PTOA can lead to the total loss of joint function, requiring joint arthroplasty to provide symptomatic relief, but these treatments are not desirable for younger patients due to the limited lifespans and their impact on quality of life.

Most drug candidates for the prevention of PTOA, such as neutralizing antibodies, IL-converting enzyme, growth factors (BMP-7, FGF-2, IGF-1), small-molecule inhibitors, lubricin or hyaluronan, aimed at slowing, halting, or reversing the progression of structural damage to cartilage have focused on mid- to late-stage OA (Lotz 2010). However, a clear precipitating injury prior to PTOA presents a unique opportunity to prevent OA initiation at an early stage. Our preliminary data, as well as several published studies, have demonstrated an encouraging chondro-protective capability of bisphosphonates (BPs) after joint injuries, which could lead to a revolution in PTOA treatment. In earlier OA studies, it was conjectured that the chondro-protective effect of BPs resulted from their inhibition on osteoclast activities (Goldring and Gravallese 2000, Reddy, Geurs et al. 2003, Sutherland, Rogers et al. 2009), yet no study has shown the direct effect of BPs on chondrocytes.

Secondly, despite the wide application of the microfracture procedure in the treatment of cartilage lesions, there is no *in vitro* model available for its mechanism study. Current studies of microfracture surgery usually require the usage of large animal models, which could require extensive resources. The ability to simulate the microfracture surgery on cartilage-bone explants, to replicate the rehabilitation procedure in bioreactors, and to longitudinally measure biomechanical and biochemical properties is obviously critical in addressing the questions raised in clinical practice. Moreover, an efficient microfracture research platform is necessary for the microfracture study to benefit from the tremendous knowledge obtained in tissue engineering and MSCs research. Thus an *in vitro* microfracture model would advance our understanding of therapeutic mechanisms of microfracture surgery, helps to develop better strategies for after-surgery rehabilitation program, and improves the outcome of microfracture treatment.

1.5 Specific Aims

Aim 1: Investigate the roles of TGF-β3 and delayed mechanical loading in the outcome of microfracture surgery.

Microfracture is an arthroscopic surgical procedure to promote the on-site repair of a cartilage lesion through the involvement of subchondral bone marrow. This minimally invasive, technically simple, and low-cost technique remains the first-line option for small cartilage lesion treatment in knee joints (Steadman, Rodkey et al. 2001, Mithoefer, Williams et al. 2006, Mithoefer, McAdams et al. 2009). However, previous clinical trials and animal studies have yielded variable and unpredictable healing results after microfracture surgery (Nehrer, Spector et al. 1999, Mithoefer, Williams et al. 2005, Kreuz, Erggelet et al. 2006). Several studies showed good short term results of the microfracture on small cartilage defects. However, soft fibrous tissue, instead of hyaline cartilage, often insufficiently filled the defect. Deterioration of new tissue could start 18 months after surgery (Kreuz, Erggelet et al. 2006). Decrease in clinical outcome was reported in several long-term follow-up studies in spite of the short-term high satisfaction of the patients (Gobbi, Nunag et al. 2005, Mithoefer, Williams et al. 2005, Kon, Gobbi et al. 2009).

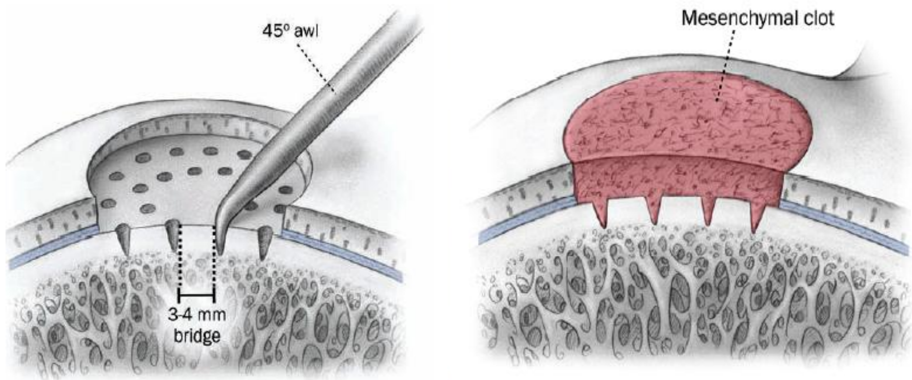

Figure 1.4 Schematic illustration depicting the fracture (left) and clothing (Bennett and Buckland-Wright) of microfracture surgery (Mithoefer, McAdams et al. 2009).

Since most studies were performed using human or large animal subjects, invasive tissue evaluation methods, such as histology, biochemical and biomechanical tests, were ethically and practically difficult to be adopted in the longitudinal assessment of repaired tissue. Therefore little is known about the precise mechanisms of tissue repair after a microfracture procedure. This hinders the improvement of surgery techniques and the search of optimized rehabilitation protocols, which are now variable and mostly empirical. No systematical studies are available on the effects of different rehabilitation protocols (Mithoefer, McAdams et al. 2009, Fazalare, Griesser et al. 2010), although abundant evidence has proved that the profiles of mechanical stimulation can regulate the proliferation, differentiation and chondrogenesis of MSCs, which is the major functional unit for tissue generation after microfracture (Awad, Wickham et al. 2004, Huang, Hagar et al. 2004).

Therefore, our first project will develop a novel *in vitro* cartilage-bone co-culture system to simulate and study the microfracture procedure. This 3D cartilage-bone co-culture model (Figure 1.5) has the advantage of allowing all involved cells (stem cells in marrow, bone cells in subchondral bone, and chondrocytes in cartilage) to maintain their *in vivo* morphology and intercellular connections, and to be surrounded by their native extracellular matrix environment. More importantly, with the ability of relative long-term culture, the functional cartilage regeneration and changes in biomechanical properties can be longitudinally assessed, which is challenging in any animal or clinical study. Using this *in vitro* culture model, we investigated the roles of chondrogenic growth factor, TGF-β3, and delayed mechanical stimulation in the outcome of microfracture procedure.

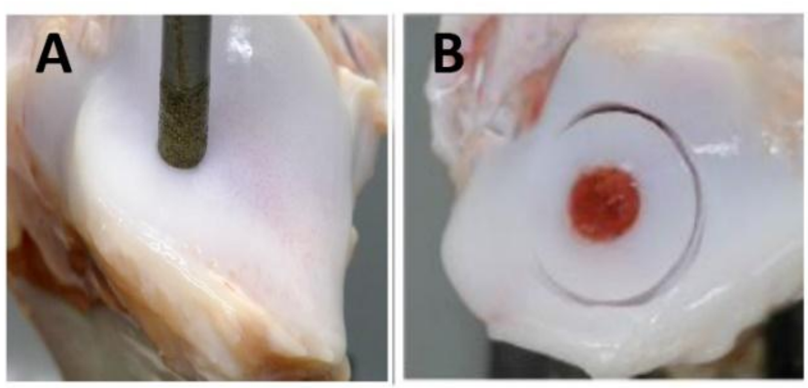

Figure 1.5 (A) Diamond tipped coring tool to drill out cartilage-bone core. (B) A cartilage-bone block with bone marrow filled in a lesion.

Aim 2: Investigate the chondroprotective mechanism of bisphosphonates and statins using the *in vitro* culture of cartilage explants.

In the progression of PTOA, bisphosphonates (BPs) treatment in cartilage has been noticed for more than a decade with its chondro-protective function. Podworny et al. found that subcutaneous injections of zoledronic acid (ZA) partially protected articular cartilage from arthritis induced by carrageenan, without affecting the induced synovitis (Podworny, Kandel et al. 1999). Zoledronic acid (ZA) is an FDA approved drug in BP family and has been widely used for the treatment of osteoclast-related bone loss (Russell 2011). In a proof-of-concept study, we also found that repeated systemic administration of ZA could suppress the development of PTOA in the DMM (destabilization of the medial meniscus) mouse model. A few similar studies, using different animal models and bisphosphonates, also noticed the chonro-protective function of BPs (Saag 2008). However, a clinical trial showed that BP cannot reverse the OA progression on patients with late stage OA (Bingham, Buckland-Wright et al. 2006). The strong influence of BPs on regular bone metabolic activities hinders their clinical applications in the young patients with joint trauma. Therefore, it is necessary to understand the chondro-protective mechanisms of BPs in the prevention of PTOA.

There are two possible mechanisms through which the BPs might suppress PTOA. One of the mechanisms is via their effects on bone remodeling, because the drug can disable the function of osteoclast, the cell resorbing bone in the human body. In most early studies, it was assumed that the chondro-protective effect of BPs resulted from their inhibition on osteoclast activities (Goldring and Gravallese 2000, Reddy, Geurs et al. 2003, Sutherland, Rogers et al. 2009). The other possibility is that ZA can directly modify chondrocyte activities, thereby preserving cartilage integrity.

In this study, we will focus on the second mechanism. Three hypotheses will be tested. First, ZA can rescue the mechanical integrity of cartilage from trauma damage *in vitro* without the presence of subchondral bone. Second, ZA can promote the beneficial effect of mechanical loading on the *in vitro* culture of cartilage explants. Third, the chondroprotective effect of ZA is related to the calcium signaling in chondrocytes. Since the BPs disable the osteoclast by inhibiting the mevalonate pathway (Fisher, Rodan et al. 2000), we further hypothesize that the effect of ZA on chondrocyte is related to its inhibition of mevalonate pathway. This hypothesis will be tested using another drug targeted at this pathway.

1.6 Organization of the Dissertation

Chapter 1 contains a brief introduction of cartilage, osteoarthritis, PTOA, prevention and treatment of PTOA. This chapter also outlines the specific aims of this thesis, and the significance of the studies.

Chapter 2 builds the *in vitro* model of microfracture surgery and studies the effect of TGF-β3 on the outcome of microfracture. To perform this study, the *in vitro* microfracture sugery model was created by harvesting calf cartilage and bone explants. A cartilage lesion was created in the center of cartilage. Bone marrow was recruited from the autologous subchondral bone. After the bone marrow was filled in the lesion, the samples were cultured for 8 weeks. The biomechanical properties, chemical composition and gene expression of the new tissue were longitudinally measured during the culture process.

In Chapter 3, different mechanical stimulation protocols were applied on the cartilage-bone explants to investigate the effects of two rehabilitation process. One is the immediate loading which involves mechanical loading on the sample a day after harvesting. The other is named delayed loading which starts the loading 2 weeks after culture. These two loading profiles simulate the immediate and delayed partial weight bearing on the knee joint after microfracture surgery.

In Chapter 4, the chondro-protective mechanism of zoledronic acid was studied. Cartilage explants from calf knee joints were cultured in serum-supplemented medium for 1 week to simulate the trauma damage on cartilage during joint bleeding. The cartilage samples were then cultured with or without the supplement of ZA for 4 weeks. Dynamic mechanical stimulation was also applied to the explants daily. The chemical composition, mechanical properties and gene expression of chondrocytes were longitudinally measured. Spontaneous calcium signaling of in situ chondrocytes were also recorded and compared to understand the chondro-protective effect of ZA.

In Chapter 5, the effect of statin was studied as a replacement of ZA. ZA inhibits the mevalonate pathway, regulates the protein prenylation (Rho, Ras), and prevents the formation of ruffle border in osteoclasts. Stain is another FDA approved drug that inhibits the mevalonate pathway. Many studies have shown that stains can promote the expression of Bone morphogenetic protein-2 (BMP-2) release, aggrecan and collagen synthesis in chondrocytes or synoviocytes, and inhibit the release of IL-1 and matrix metalloproteinase 3 (MMP3). Cartilage explants were treated with different concentration of either atorvastatin or simvastatin after trauma damage induced by serum or interleukin-1α. The mechanical properties were measured over time with the treatment.

In Chapter 6, summary of all the studies are presented. Potential future directions based on current results are also discussed.

Chapter 2

A LONG-TERM IN VITRO CULTURE MODEL OF MICROFRACTURE SURGERY

2.1 Introduction

2.1.1 Microfracture Surgery

Microfracture is a widely used arthroscopic surgical procedure for the repair of cartilage lesions (Steadman, Rodkey et al. 2001, Steadman, Briggs et al. 2003, Mithoefer, Williams et al. 2006). This surgical technique is minimally invasive, technically simple, low-cost, and is considered as the first-line option for small cartilage lesion treatment in knee joints. In the treatment, damaged cartilage is first drilled or removed until the underlying bone is exposed, then the subchondral bone is perforated for the release of bone marrow and formation of a blood clot within the lesion site (Figure 2.1). New cartilaginous tissue is regenerated by the cells within the bone marrow (*e.g.*, mesenchymal stem cells).

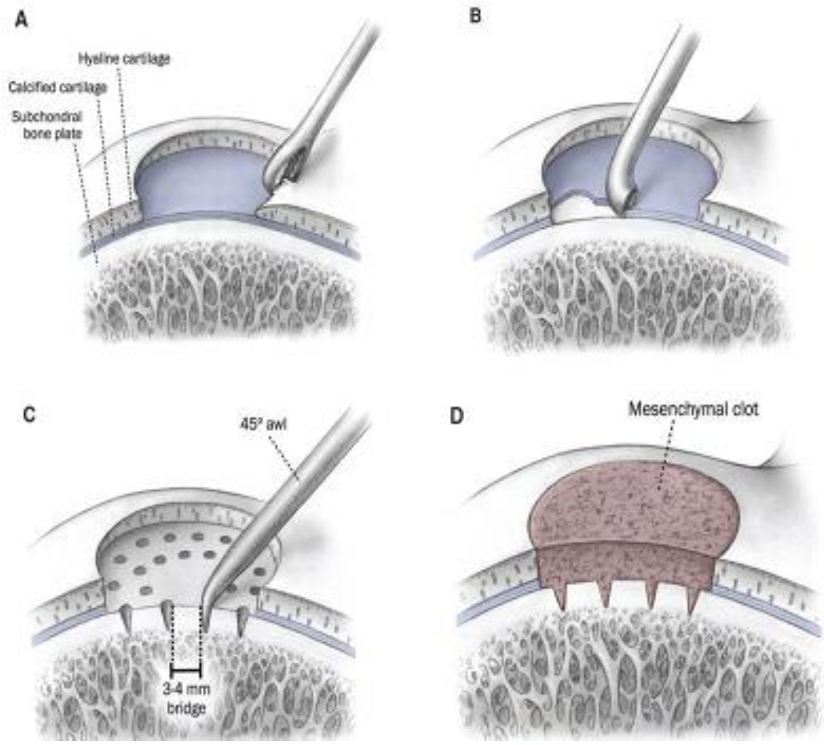

Figure 2.1 Microfracture technique. (A) Debridement. (B) Removal of calcified cartilage. (C) Microfracture penetration. (D) Anchored mesenchymal clot. (Adopted form Mithoefer et. al).

There are several studies that have shown the outcome of microfracture technique as offering clinical, functional and subjective improvement in athletically active patients (Knutsen, Engebretsen et al. 2004, Gobbi, Nunag et al. 2005, Cerynik, Lewullis et al. 2009, Frank, Van Thiel et al. 2010). One of the follow-up studies after microfracture by Van Assche et al. was performed on the small lesions sized 1 to 5 cm^2 at 6 months and 2 years (Van Assche, Van Caspel et al. 2009, Van Assche, Staes et al. 2010). The study showed that 17 of 26 patients showed significant improvements in functional performances. A few studies have also shown that the microfracture treatment to nonathletic adults with small lesions showed statistically significant improvements in clinical outcome scores at 3 years postoperatively (Saris, Vanlauwe et al. 2008, Vanlauwe, Saris et al. 2011, Lim, Bae et al. 2012) .

Microfracture works best for small lesions (diameter = 1-2.5 cm) of cartilage damage (Knutsen, Engebretsen et al. 2004, Jones and Peterson 2006, Kreuz, Steinwachs et al. 2006, Bekkers, Inklaar et al. 2009). Patients with under 40 years old might have intrinsically superior healing responses than older patients (Kreuz, Erggelet et al. 2006). The optimal results from the microfracture have been observed on patient with a body mass index less than 30 and experience symptoms (activity related pain, swelling, locking, and catching) for less than 1 year. However, some clinical trials and animal studies have yielded variable and unpredictable healing responses after microfracture surgery (Gill and Macgillivray 2001, Gudas, Kalesinskas et al. 2005, Mithoefer, McAdams et al. 2009, Von Keudell, Atzwanger et al. 2012).

2.1.2 Challenges of Microfracture

The newly generated tissue after microfracture is often regarded as scar tissue or fibrocartilage (Gill and Macgillivray 2001, Steadman, Briggs et al. 2003). The fibrocartilage is suitable to resist tension forces, but unable to withstand long-term variable cyclic load and shearing forces, while the hyaline cartilage is perfect to sustain compression forces. Therefore the repaired tissue after microfracture is much weaker than natural hyaline cartilage and displays poor long-term performance due to poor compressive strength and low durability (Beris, Lykissas et al. 2005). The new scar tissue contains mainly type I collagen and less proteoglycans when compared to native articular cartilage, resulting in inferior biomechanical properties. Improvements on microfracture procedure have always been desirable to optimize the surgery outcome.

The fundamental mechanism of microfracture is to stimulate the formation of cartilaginous tissue by creating a clot populated with growth factors, platelets and bone marrow derived progenitor cells capable of chondrogenesis. MSCs are considered to be the most capable cell population in the clot for the generation of new cartilage. As a particular ideal cell source for cartilage tissue engineering, MSC, as well as its capability to generate neocartilage, has been widely investigated, especially for potential applications in ACI (Baksh, Song et al. 2004, Huang, Stein et al. 2009, Huang, Farrell et al. 2010). However, mechanical properties of the constructs populated by MSCs are typically inferior to both native tissue and to tissue-engineered constructs using chondrocytes (Huang, Stein et al. 2009). Chondrogenic differentiation of MSCs has been challenging and requires proper environment, biochemicals, culture length, and mechanical stimulation. MSCs are often inclined to osteogenic differentiation, which attributes to the formation of fibrous cartilage at the lesion site after microfracture treatment (Frisbie, Trotter et al. 1999). Therefore, to promote the generation of functional hyaline cartilage, it is critical to promote the MSC chondrogenesis in the marrow clot.

2.1.3 Growth Factors and Chondrogenesis

Approaches to promote the chondrogenesis in MSCs include appropriate pellet/matrix composition, chondrogenic differentiation supplements, low oxygen tension, and some forms of mechanical load. Several chondrogenic growth factors, specifically those in the transforming growth factor β (TGFβ) super family, have been identified. These include TGFβ-1, -2, and -3, and bone morphogenetic proteins (BMPs)-2, -4, -6, -7, and -9. In particular, TGF-β has been used for decades in *in vitro* assays of cartilage micromasses or pellets starting from culture-expanded MSCs

(Mackay *et al.*, 1998; Pittenger *et al.*, 1999), and has become a requirement for chondrogenesis. TGF-β initiates cartilage-specific gene expression through intracellular signaling cascades (Lutz and Knaus, 2002; Schmierer and Hill, 2007). There were also studies introducing growth factors into microfracture to obtain hyaline-like cartilage. One study related the effect of TGF-β3 with microfracture. This study focused on the effect of synovial fluid with the progenitor cells stimulated with TGF-β3, and the results suggested that synovial fluid could impair the chondrogenic development of mesenchymal progenitors in microfracture (Kruger, Endres et al. 2012).

BMP-2 is another popular supplement chosen to induce differentiation of bone-marrow-derived MSCs into osteoblast and chondrocyte differentiation (Yang, La et al. 2011). Long term delivery of the BMP-2, using heparin-conjugated fibrin as carriers, was tested at rabbit articular cartilage defects treated with microfracture. After 8 weeks, microfracture with BMP-2 resulted with more GAG and type II collagen contents, and thus better hyaline cartilage than the control groups. Another study performed by Kuo et al. supplemented BMP-7 in a collagen sponge at the lesion site in rabbit joints (Kuo, Rodrigo et al. 2006). The BMP-7 combined with microfracture acted synergistically to stimulate the cartilage repair, and generated more repair tissue similar to native hyaline articular cartilage.

A dedicated study has compared the chondrogenic effectiveness of different growth factors, including TGF-β3, IGF-1 and BMP-7 (Indrawattana et. al, 2004). It was shown that TGF-β3 could bring the most chondrogenic features among the single use of those growth factors. As TGF-β3 is the most widely used growth factor for the in vitro chondrogenic induction of MSCs (Mauck, Byers et al. 2007) , it will be employed in this study to promote the growth of new cartilage from bone marrow.

2.1.4 Aims and Significance

The first aim of this study is to develop an *in vitro* 3D cartilage-bone co-culture model to mimic the microfracture surgery. The ability to reproduce the microfracture surgery on cartilage-bone explants could be critical in addressing the questions raised in clinical practice. Previous microfracture studies have been mainly using human or large animal subjects, thus traditional tissue evaluation methods such as histology, biochemical, and biomechanical tests have been ethically and practically difficult to include in a longitudinal assessment of the repaired tissue at the lesion site. An *in vitro* culture system could easily overcome this limitation. Moreover, an efficient microfracture simulation platform is necessary for the microfracture to benefit from the tremendous knowledge obtained in cartilage tissue engineering and stem cell research, such as the use of growth factors, new biomaterials and mechanical stimulations.

The second aim of this chapter is to study the effect of the growth factor, TGF-β3, on the outcome of microfracture using the newly developed *in vitro* microfracture system. Results of this study will provide information about the sensitivity of the *in vitro* system, viability of the MSCs in bone marrow, and roles of the MSCs in tissue repair. It helps to advance our understanding of therapeutic mechanisms of microfracture surgery and the search of improvement technique of the surgery.

2.2 Methods

2.2.1 Cartilage-Bone Core Harvesting and Microfracture

Cartilage-bone cores were harvested from 3-month-old calf knee joints (Green Village, NJ). After opening the calf joint in a sterile biological safety hood, a cylinder of cartilage-bone core (diameter =8 mm) was removed from the central region of tibia (Figure 2.2) with a diamond tipped coring tool (Starlite Industries, PA). The height of the cartilage-bone core was cut to 7.5 cm with an isomet low-speed saw (Buehler, IL). All drilling or cutting was constantly irrigated with phosphate buffered saline (PBS) (Figure 2.2). The cartilage layer on the tibia was approximately 2-4 mm in thickness, and the rest was subchondral and trabecular bone. The trabecular bone on the explant was cleaned of bone marrow with sterile PBS using an Interplak water pick (Conair Corp, CT). A full thickness 3 mm lesion in the center of cartilage was created with a biopsy punch, and the calcified cartilage layer was also carefully removed. Bone marrow harvested from the same joint (Figure 2.3E) was filled at the lesion site (Figure 2.3D). All explants were move to 6-well Petri dish and cultured for 7 weeks in *Group 1: Control Medium* (Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin) or *Group 2: Chondrogenic Medium* (DMEM with 10% FBS and 1% Insulin-Transferrin-Selenium (ITS), 0.1 μM dexamethasone, 1 mM sodium pyruvate supplemented with ascorbate acid (50 μg/ml) and 10 ng/ml of Transforming growth factor beta 3 (TGF-β3) (Bian, Zhai et al. 2011).

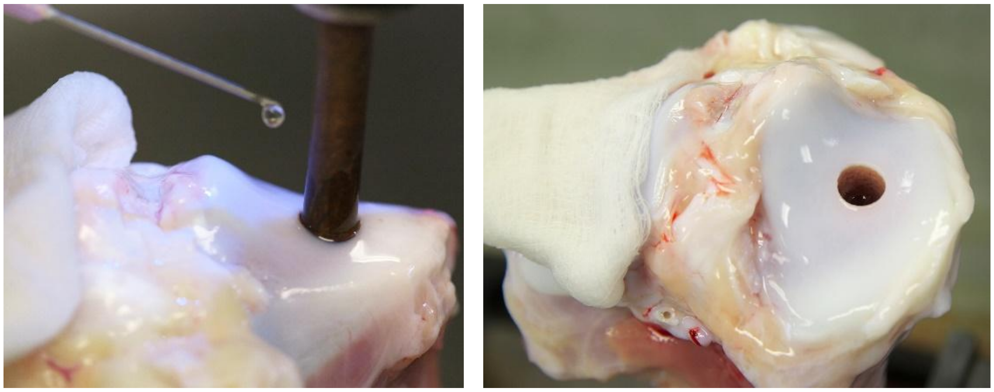

Figure 2.2 (Left) Drilling for cartilage-bone explant (Gerszten, Albright et al. , Chan, Lu et al. 2009): The cylinder was harvested from the center region of the calf tibia plateau.

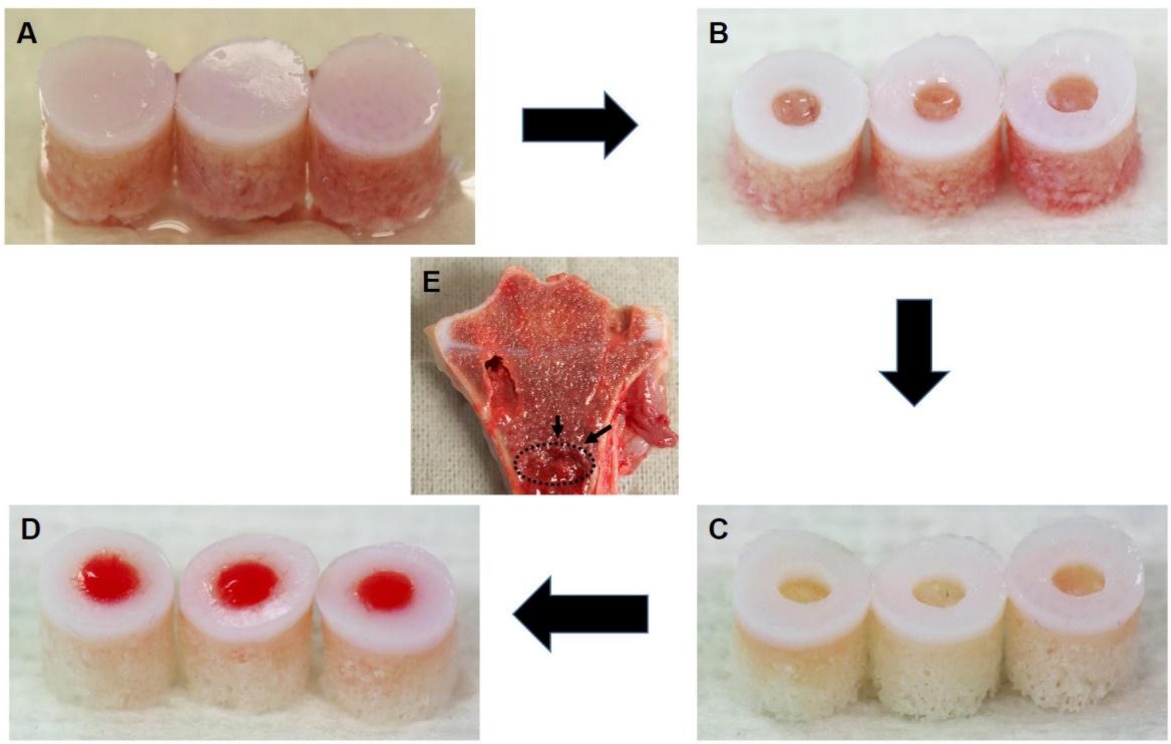

Figure 2.3 Microfracture procedure was described in an order from (A) to (D). Image (E) describes the source region of bone marrow.

2.2.2 Mechanical Testing

To determine the mechanical properties of newly-formed tissue at lesion site, the cartilage-bone block was mounted onto a custom-built micro-indentation device for indentation testing as described previously (Lu, Mow et al. 2009). A rigid flat-ended porous-permeable indenter tip (diameter=1.6mm) was used to perform indentation stress-relaxation on the newly formed tissue. A 2 gram tare load was applied on the sample to locate the surface, followed by indentation to a depth of 200 μm at a ramp speed of 1um/s. The constant deformation was then maintained until equilibrium was achieved. The instantaneous and equilibrium Young's moduli of the tissue were determined from the force response curve, using the elastic indentation solution developed for cartilage by Hayes et al (Hayes, Keer et al. 1972). The indentation testing was performed longitudinally at weeks 2, 4, and 7 on 6 samples per group.

2.2.3 Histology

After 7-week culture, two explants were fixed in 4% paraformaldehyde and then incubated in 70% alcohol. The specimen was then decalcified in 10% buffered formic acid, paraffin embedded, and sectioned at 5 μm-thick slabs such that full-depth cartilage-bone sections were obtained. Sections were stained with Hematoxylin and Eosin (H&E), Picro Sirius red and Safranin-O (Mauck, Nicoll et al. 2003). Type I and type II collagen were stained with immunohistochemistry technique (Huang, Yeger-McKeever et al. 2008). The sections were first deparaffinized and rehydrated. Antigen retrieval was performed in proteinase K (0.02 ml/ml) for 30 min at 37 °C. Sections were then incubated in 0.5 M acetic acid to swell tissue for 4 hours at 4 °C. Using the serum-free commercial protein block (Dako, X0909), slides were incubated for 10 min in 4 °C, and then primary collagen antibody (1:250 for Collagen I (Abcam, ab138492) and 1:100 for Collagen II (Abcam, ab 21921) in blocking solution) was added on top of samples and incubated for overnight at 4 °C. Secondary antibody in blocking solution was prepared in 1:100 concentration and incubated for 1 hour at 4 °C. The fluorescent slides were imaged using Zeiss confocal microscope (Zeiss LSM510, Carl Zeiss Inc., Thronwood, NY).

2.2.4 Cell viability

After 7-week culture, two samples were washed with PBS and first stained in 4 µM Ethidium homodimer (EthD-1) medium for 30 min at 37 °C to dye the dead cells. Cartilage-bone explant was then cut into two halves using a custom-built cutting tool and washed with PBS for 3 times. Samples were then incubated in 1.5 µM calcein-AM (Molecular Probes, Inc) for 45 min at 37 °C to dye the live cells. After three more PBS washes, constructs were imaged using a one photon confocal microscope (Zeiss LSM510, Carl Zeiss Inc., Thronwood, NY).

2.2.5 Biochemical Assays for GAG and Collagen Contents

After mechanical testing at day 43, the tissue plugs at the lesion site were harvested for biochemical assay. To measure the GAG and collagen contents, the tissue was digested overnight at 60 ^{0}C with the digestion buffer (0.1 M Sodium acetate, 10 mM of Cysteine HCL and 50 mM EDTA) and 20 µl/ml papain. GAG concentration in the digested samples was assayed using the Blyscan Glycosaminoglycan Assay kit (Accurate Chemical & Scientific Corp., Westbury, NY). Chondroitin-4-sulfate was used as standard. Briefly, samples were incubated with the dye reagent. The GAG-dye complex was precipitated by centrifugation. After the supernatant was completely drained, the precipitate was re-suspended in dye dissociation reagent, and absorbance was read on a SpectraMax 190 (Molecular Devices) plate reader at 620 nm. To measure collagen content, the digested samples were mixed with 12 N HCl for 1:1 concentration and incubated at 100 °C overnight. The samples were taken out of the oven and placed in a vacuum oven for acid neutralization, during which the samples were incubated at 56 °C until no moisture was present. Stock buffer (0.238 M Citric acid monohydrate, 0.012 (v/v) Glacial acetic acid, 0.88 M Sodium acetate trihydrate, 0.85 M Sodium hydroxide in deionized water) was used to resuspend the sample. The resuspended samples were first incubated in chloramine T for 20 min at room temperature. Later, P-dimethylaminobenzaldehyde (DMAB) diluted in propanol were added into the sample in the 60 °C waterbath for 7 mins. The absorbance was read on a SpectraMax 190 plate reader at 540 nm.

2.2.6 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The expression levels of aggrecan, type I and type II collagen of cells at the lesion site were measured using qRT-PCR on 6 samples per group at weeks 2, 4, and 7. Tissue at the lesion site was taken out of the cartilage-bone construct and digested in a Trizol (Invitrogen)/chloroform (5:1 v/v) mixture. The digestion mixture phase was separated into three layers: an aqueous RNA layer (top), a middle DNA layer, and a bottom organic layer containing total proteins. The top RNA layer was carefully aspirated and retrieved from the aqueous phase by precipitation in isopropanol, followed by centrifugation (14,000 rpm, 15 min) and thorough washing (twice with 75% ethanol) before being reconstituted in RNase-free H2O (Invitrogen). The RNA concentration was determined using a Nanodrop ND-1000 Spectrophotometer

(Nanodrop Technologies). RNA samples were reverse transcribed into cDNA using a QuantiTect Reverse Transcription kit (Qiagen). Sequence-specific amplification from cDNA was performed and quantitatively detected by an ABI 7300 real-time PCR system using SYBR green PCR master mix (Applied Biosystems). PCR thermal cycle was programmed into three stages: initialization at 95 °C for 10 min, denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min (total of 45 cycles). The purity of the PCR product was confirmed by the dissociation curve, constructed by sequential heating of the product (95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s). The expression of collagen type I (COL I), collagen type II (COL II) and aggrecan (ACAN) genes were evaluated with the sequences listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house keeping gene. All primers were synthesized by Life Technologies. The qPCR data was analyzed by qbasePLUS software (V2.5; Biogazelle, Ghent, Belgium). Relative expression (fold change) of the gene of interest was normalized by GAPDH and further normalized by the gene expression of 2 week control sample.

Table 2.1 Sequences for the primers used in qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Gene Bank no.	Product size (bp)
ACAN	CCTGAACGACAAGACCATCGA	TGGCAAAGAAGTTGTCAGGCT	NM_001135.3	101
COL I	CTTCTGGAGCAAGTGGTGAA	GATCCTTCAGCACCAGGAG	XM_005257059.1	109
COL II	GCATTGCCTACCTGGACGAA	GAACCTGCTGTTGCCCTCAG	NM_001844.4	101
GAPDH	GCTGGTGCTGAGTATGTGGT	CAGAAGGTGCAGAGATGATGA	XM_005253678.1	109

2.2.7 Statistical Analysis

A 2-way ANOVA with Bonferroni post-hoc analysis (or Scheirer-Ray-Hare test, if non-parametric) was used to compare the mechanical properties, gene expression levels, GAG and collagen contents with effects for chondrogenic medium. Linear regression will be used to examine the time-dependence of the outcome parameters. All data is presented as mean ± standard deviation. Statistical significance was established at $p < 0.05$.

2.3 Results

2.3.1 Co-culture of Cartilage-bone Explant

The cell viability was first checked to investigate the feasibility of the long-term *in vitro* culture of cartilage-bone explants. Since many previous studies have proved the feasibility of long-term culture of cartilage explants alone, we aimed to identify the effects of the presence of bone during cartilage culture. Three *in vitro* culture groups were designed, including cartilage alone, cartilage with separated bone explant, and cartilage-bone explant from calf knee joints. After 28 days *in vitro* culture (medium: DMEM with 10% FBS), live/dead stain of the chondrocytes in cartilage showed no difference between three groups. Most chondrocytes in cartilage are alive in all three groups (Figure 2.4). Histology pictures of Safranin-O staining showed that the cartilage only culture has less GAG content than cartilage in the other two groups (Figure 2.4). Therefore, the co-culture with bone has no adverse effects on chondrocytes viability in cartilage, and the presence of bone even showed beneficial effects on the GAG content of cartilage explant.

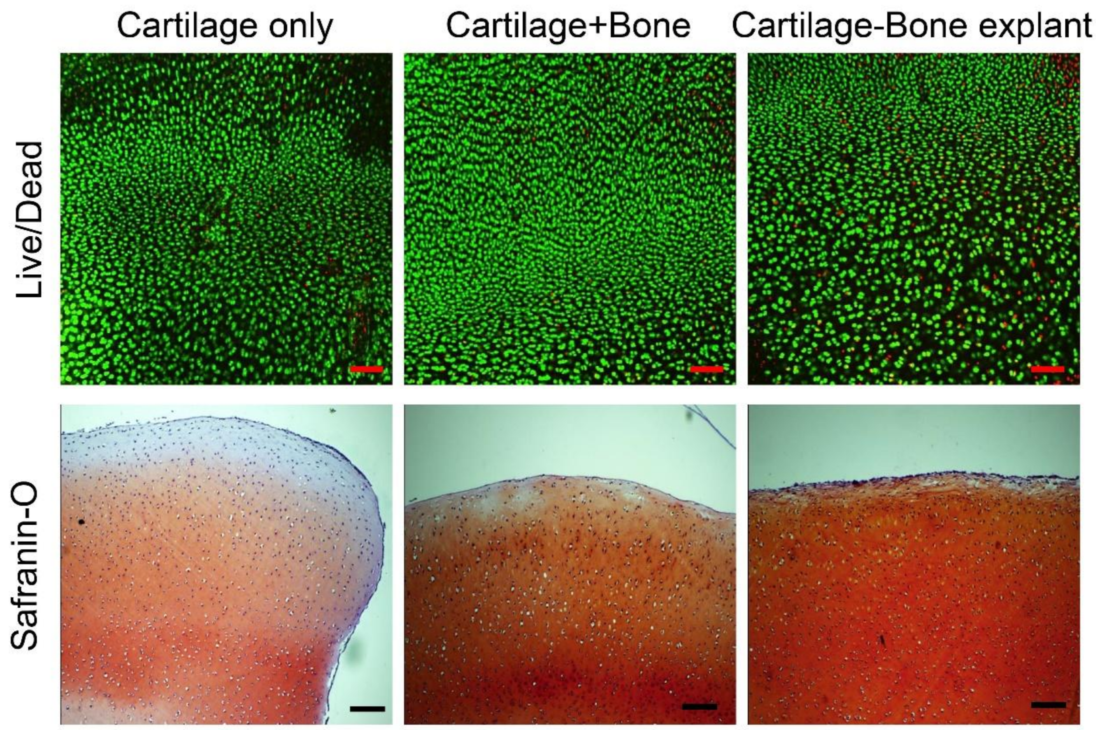

Figure 2.4 (Top) Live/Dead stain of chondrocytes in cartilage cultured with or without the presence of bone. (Bottom) Safranin-O staining of cartilage after 28 days culture. (Red scale bars = 100 µm, Black scale bars = 200 µm).

2.3.2 Effect of Chondrogenic Medium Supplemented with TGF-β3

At the end of the 7 week culture, cell viability in surrounding cartilage, newly generated tissue at lesion site, and subchondral bone was checked with Live/Dead staining. Most of the cells in the surrounding cartilage and bone tissue were alive in both Control and Chon groups, while the cartilage in Chon group showed less dead cells than the control group. However, a large amount of dead cells existed at the lesion site, and the control group had more dead cells than the Chon group (Figure 2.5).

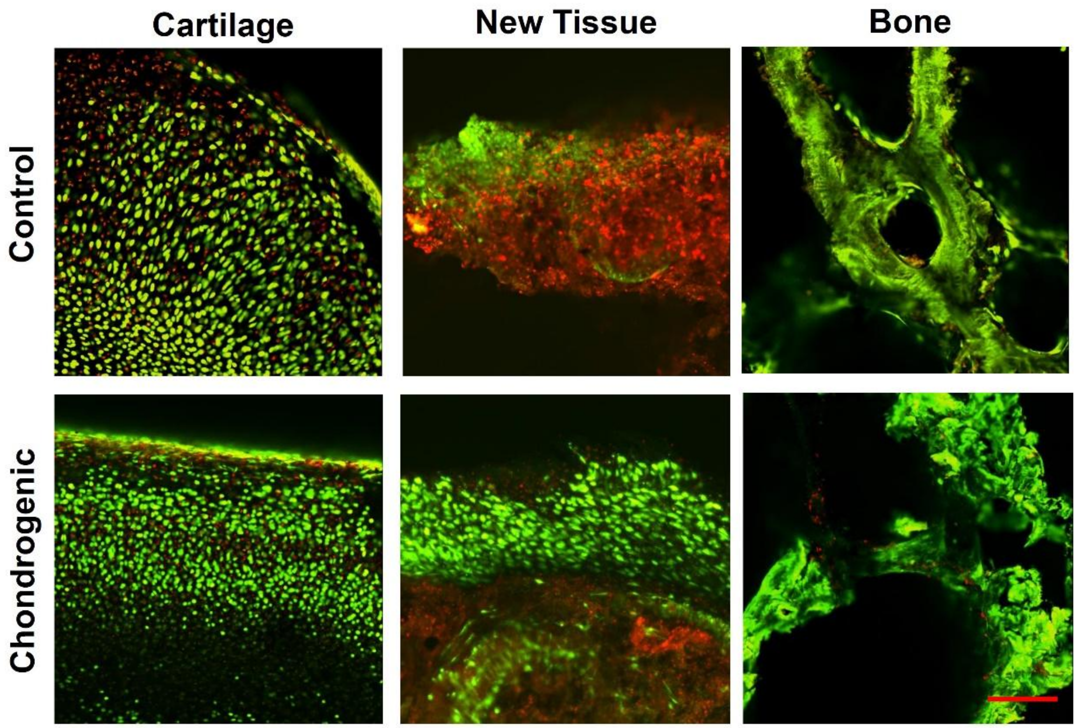

Figure 2.5 Live/Dead staining to visualize cell viability in cartilage, tissue at lesion site, and subchondral bone after 7 weeks culture in control (top) or chondrogenic (bottom) medium (Scale bar= 200 µm).

At the lesion site, the bone marrow formed clot collapsed significantly and can barely fill half of the lesion after 7-week culture in control medium (Figure 2.6). In contrast, sample cultured in chondrogenic medium showed better outcome with new connective tissue covering the entire lesion surface. In the superficial zone, the newly formed cartilaginous tissue integrated with the surrounding cartilage, although such integration is missing in the middle-deep zone. In the deep zone, the newly formed tissue failed to integrate with the subchondral bone, leaving a gap on top of the bone. In the Chon group, collagen and GAG content were observed from the Safranin-O and Picosirius Red staining, especially in the top layer. However, it is easy to note that the collagen and GAG contents in the repaired tissue are much lower than those of the surrounding cartilage.

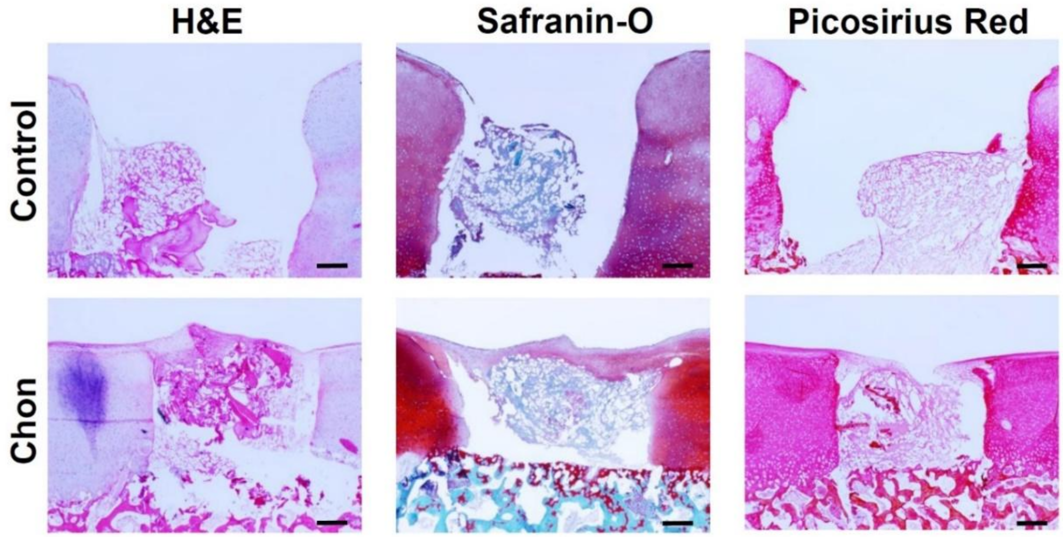

Figure 2.6 Histological images of repaired tissue, surrounding cartilage and subchondral bone (scale bar = 200 µm).

GAG content of the new tissue cultured in the Chon medium was much higher than the control group at 2 week (1.28 ± 0.37% vs. 2.76 ± 0.37%) and 4 week (1.30 ± 0.25% vs. 2.9 ± 0.22%) culture (Figure 2.7A). No significant difference was detected in collagen content between two groups during the culture period (Figure 2.7B). Instantaneous modulus of the new tissue at 2 week was 1.43 ± 0.25 kPa for the control, significantly lower than the Chon group (1.83 ± 0.58 kPa). After 7 week culture, the instantaneous modulus was still significantly higher in the Chon group (3.05 ± 0.37 kPa vs. 2.16 ± 0.15 kPa) (Figure 2.7C). The equilibrium Young's modulus increased over time for both groups, and the Chon group became significantly higher than the control group after 7 week culture (1.82 ± 0.38 kPa vs. 1.02 ± 0.37 kPa) (Figure 2.7D). Immunohistochemical images detected little type I collagen content at the lesion site for both groups (Figure 2.8 A-B). The new tissue in the Chon group, especially the superficial layer, showed significant staining of type II collagen (Figure 2.8D).

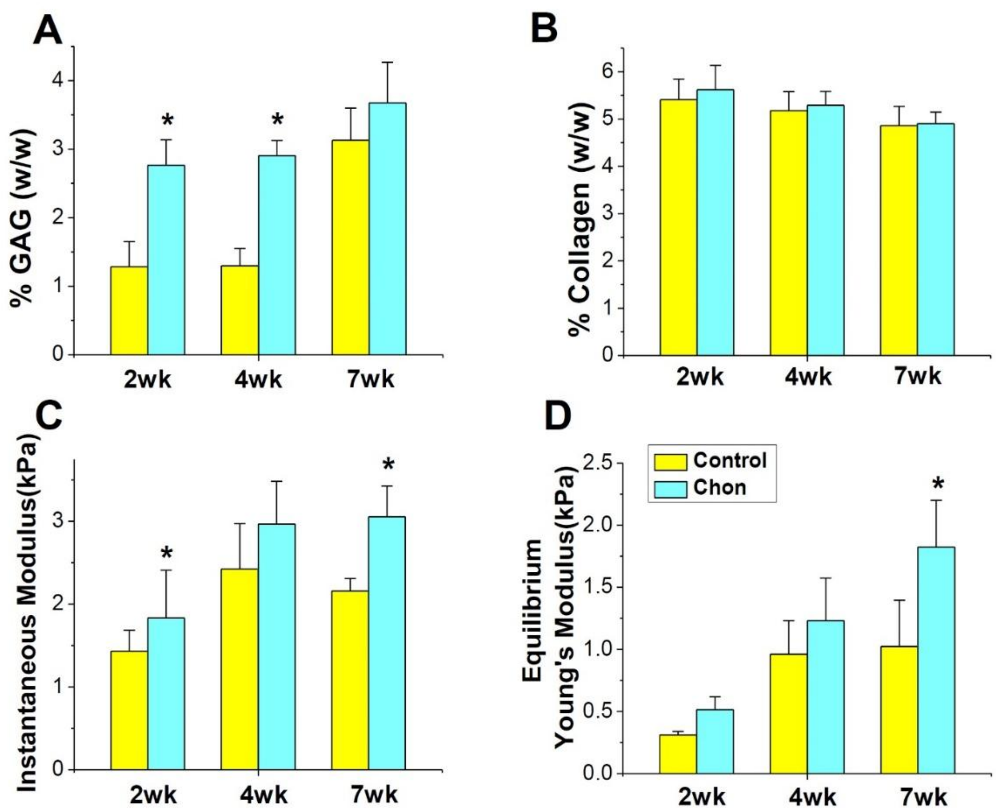

Figure 2.7 (A) GAG contents and (B) Collagen contents at 2, 4 and 7 week time points. (C) Instantaneous modulus and (D) Equilibrium young's modulus measured by micro-indentation test. (n=6; *: $p<0.05$, error bars: standard deviation).

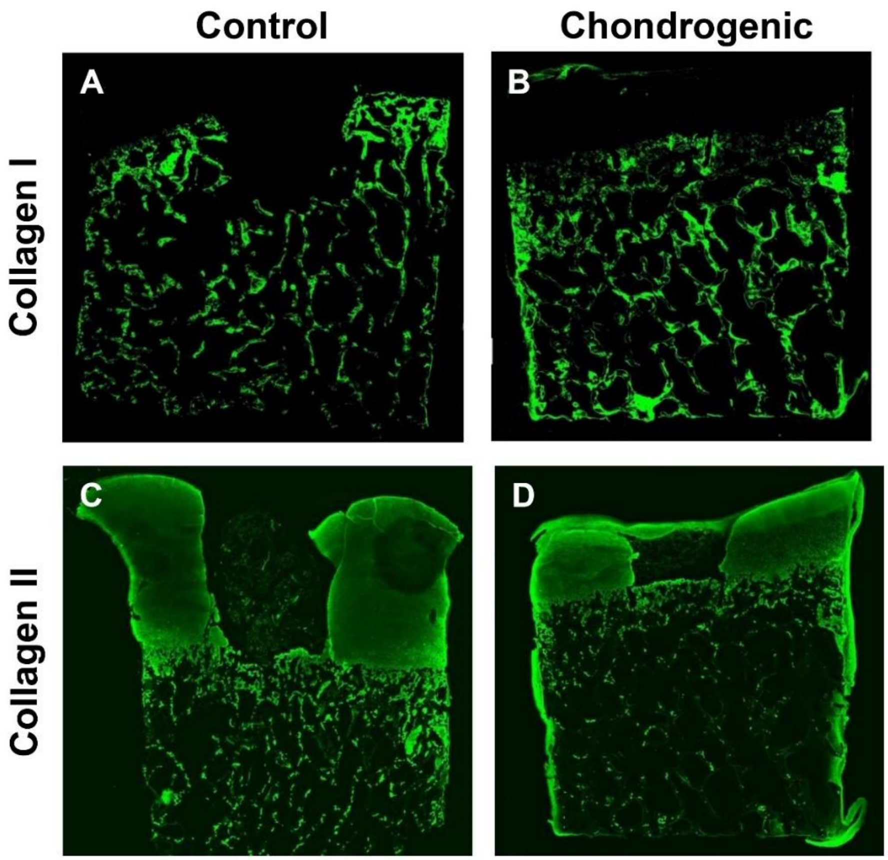

Figure 2.8 Immunohistochemical images of Type I (A, B) and II (C, D) Collagen.

Expressions of aggrecan, type I and type II collagen genes in the cells at lesion site are measure longitudinally during the culture. The expression levels were normalized to that of control at week 2. In the chon group, the expression of type I collagen gene decreased over time (Figure 2.9A), but showed no variation in the control group. At week 2, chondrogenic group had almost 5 times more expression of type I collagen than the control. Although the Chon group is still higher than the control at week 7, the gap between groups has decreased significantly. Collagen II expression was significantly higher in the Chon group at week 4 (1.299 ± 0.1625 vs.

1.821 ± 0.3843) and week 7 (2.717 ± 0.835 vs. 4.677 ± 1.221) (Figure 2.10B). For both groups, the expression of type II collagen increased with the culture time, as well as the aggrecan gene expression (Figure 2.9C). The aggrecan expression was always higher in the Chon group during 7 weeks culture (week 2: 1 vs 2.677 ± 0 .879; week 4: 3.553 ± 0.304 vs 9.622 ± 1.583; week 7: 7.14 ± 0.861 vs 10.5 ± 1.367).

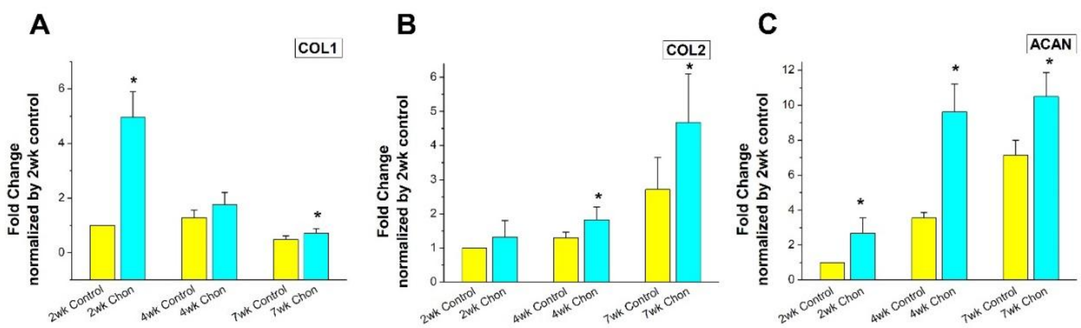

Figure 2.9 Gene expression level by qRT-PCR. (A) Type I collagen, (B) Type II collagen, (C) Aggrecan. (Normalized to GAPDH and then the level of control group at week 2) (n=6; *: p<0.05 error bars: standard deviation).

2.4 Discussion

Using a 3D *in vitro* culture system, the microfracture surgery was successfully simulated on cartilage-bone explants. New cartilaginous tissue was generated at the lesion site from bone marrow. In this co-culture system, the *in vivo* morphology and intercellular connections of bone marrow cells with bone and cartilage cells were maintained, while avoiding the complexities of *in vivo* systems so that the roles of individual cell types, single biomechanical and biochemical factors can be investigated independently (de Vries-van Melle, Mandl et al. 2012). Most importantly, with the ability of long-term culture, the functional cartilage regeneration and changes in biomechanical properties can be longitudinally assessed, which could be challenging in animal or clinical studies.

It is generally believed that bone marrow MSCs are the major contributor for the generation of new cartilage at the lesion site (Huey, Hu et al. 2012). Usage of bone marrow derived MSCs as the major cell source has been extensively studied in cartilage tissue engineering research (Johnstone, Hering et al. 1998, Sekiya, Vuoristo et al. 2002, Wakitani, Imoto et al. 2002). Although many evidence showed that MSCs can differentiate into chondrocytes and generate neocartilage, the chondrogenesis of MSCs requires special niche, such as 3D culture environment, proper growth factors and physical stimulation (Mauck, Yuan et al. 2006). Most *in vitro* studies showed that neocartilage generated by MSCs are mechanically inferior to those from mature chondrocytes (Mauck, Yuan et al. 2006, Erickson, Huang et al. 2009, Huang, Stein et al. 2009). In microfracture, the bone marrow MSCs are embedded in the clot. The nutrient supply for regular chondrocytes is mainly from synovial fluid and the nutritional support are deprived (Ytrehus, Carlson et al. 2007, Wang, Wei et al. 2013). The porosity of bone marrow clot could be much lower than the native cartilage. Therefore, it is not a surprise that the MSCs in the clot have very limited nutrient supply. This hinders the chondrogenesis of MSCs and their synthesis of cartilage ECM, which could be attributable to the formation of fibrous cartilage with low mechanical strength at the lesion site. As a major surgical technique for cartilage repair, microfracture attracted many studies using animal model or clinical trial (Breinan, Martin et al. 2000, Steadman, Rodkey et al. 2002). Our study developed the first *in vitro* model to simulate the microfracture process. A difficulty with this system was to have bone marrow settle at the lesion site and to maintain the cell viability in the clot. The marrow can easily float away from the lesion site. Medium was changed by switching each sample into a well with new medium to avoid hydraulic pressure. However, the low porosity of the bone marrow, which is mainly composed of fat, still prohibits the nutrient supply to residing MSCs. Our results clearly showed that the superficial layer has much higher GAG content and collagen fibers than the middle-deep zone, which proves the importance of nutrient supply for the generation of new cartilage. Moreover, the generation of neocartilage in our current *in vitro* system is not necessarily slower than the natural *in vivo* process, because the culture medium may provide the cells with more nutrients and growth factors than the synovial fluids. In clinical study, it is generally believed that the new cartilage can be formed half year after the surgery (Steadman, Rodkey et al. 2001). In this study, the neocartilage can cover the lesion surface in 2 months, which could be a result of the abundant nutrients in medium. Finally, the high collagen and GAG contents in the top layer provide a false visual impression that the lesion is filled with cartilaginous tissue, while the tissue in the middle-deep zone are much weaker than the surface tissue. This could mislead the arthroscopic examination during clinical practice.

TGF-β3 is a major growth factor employed for the generation of neocartilage. It can inhibit the de-differentiation of chondrocytes, promote the chondrogenesis of MSCs, improve the synthesis rate of ECM, and prevent the hypertrophy of chondrocytes (Johnstone, Hering et al. 1998). In this study, it is clear that the supplement of TGF-β3 benefits the outcome of microfracture significantly. Indeed, the bone marrow can barely attach to the surrounding cartilage in control medium and could float away during the culture. After 7-week culture in control medium, the bone marrow also collapsed with much smaller volume. In contrast, the supplement of

TGF-β3 in medium promoted the integration of bone marrow with native cartilage and maintained the volume of the clot. Vision inspection of the articular surface reveals that the lesion site is fully covered by neocartilage. At a few time points, the GAG and collagen contents of control groups are not significantly lower than the Chon group. This could be related to the small volume of the new cartilage in the control group. A collapsed small clot could have much more efficient transport for the cells. Nevertheless, our results clearly proved that supplement of TGF-β3 generated better neocartilage at the lesion site. This demonstrates that the cells in bone marrow, during *in vitro* culture, can respond to the growth factors with the generation of cartilage ECM. This also implies that current microfracture model could be used to study the effects of individual factor on the outcome of microfracture. According to the results, supplement of TGF-β3 to the cells in the bone marrow clot may significantly improve the surgical outcome, which could be achieved by local delivery with biochemical carriers.

In the present microfracture model, subchondral bone was cultured together with the cartilage. When compared to the cartilage O-ring system used for cartilage tissue engineering research (Jaasma, Plunkett et al. 2008), the co-culture with subchondral bone could increase the complicacy of the system. It has been shown that co-culture with bone cells can modify the metabolic activities of chondrocytes (Spalazzi, Dionisio et al. 2003). However, removal of the cartilage from the subchondral bone can change the pre-stress inside the tissue and further alter its mechanical properties. Presence of the bone is closer to the *in vivo* situation. Since it is difficult to maintain the viability of bone marrow located in trabecular bone explant (Chan, Lu et al. 2009), bone marrow was removed in the trabecular bone. The major cell population left in the bone explant should be osteoblasts, bone surface cells and osteocytes. The live/dead stain revealed a high density of live cells inside the porous bone after 7-week culture. As demonstrated in the preliminary study, the presence of bone cells may affect the chondrocyte activities. Co-culture with the subchondral bone also revealed the insufficient nutrient supply to bone marrow cells close to the bone. The new tissue can barely integrate with the porous bone. It has been shown that the fracture on bone surface can barely be repaired after the surgery, but it is not clear whether the exposing of bone surface could improve the nutrient supply to the cells in the clot. As no study has revealed angiogenesis of the repaired cartilage after microfracture, it is very likely that the scar tissue formed at the bone surface blocked the transport between the clot and porous bone. Therefore, the repaired cartilage in the deep layer could also be weaker due to the lack of nutrient supply, as revealed in the present study. Enhanced nutrient supply to cells in the bone marrow clot could be a major direction to improve the outcome of microfracture surgery.

In the immunohistochemistry images, some type II collagen was stained in the subchondral bone area besides abundant type I collagen. As the collagen in bone should be mainly type I, the type II collagen observed here should be formed during the long term in vitro culture. When the osteochondral explants were prepared, the bone marrow in subchondral bone was removed with water jet, which may not be able thoroughly remove the progenitor cells attached on the bone surfaces. The remaining bone marrow and bone surface cells, especially those trapped in the calcified cartilage-bone interface, will differentiate into chondrocytes and generate cartilaginous tissue.

2.5 Conclusions

In this study, we demonstrated the feasibility of simulating microfracture surgery using an *in vitro* osteochondral explant model. This model could be a powerful platform to study the interactions between bone marrow, cartilage and bone cell networks and to test new techniques to improve the surgery outcome. Using this explant model, we found that supplement of TGF-β3 significantly increased the volume, stiffness, GAG and collagen contents of the new tissue. In the top layer of new cartilage, a dense type II collagen network was generated and integrated with the native cartilage, which may be attributed to the sufficient exposure of bone marrow cells to culture medium. Our results also indicate that a localized long-term delivery of TGF-β3 to the bone marrow could significantly promote the generation and integration of new repair tissue after microfracture surgery.

Chapter 3

DELAYED MECHANICAL LOADING FOR THE REHABILITATION OF MICROFRACTURE SURGERY

3.1 Introduction

Rehabilitation protocols are believed to be crucial for the outcome of the microfracture procedure. An optimized rehabilitation protocol should consider many factors and be patient-specific (Table 3.1). Factors such as specific time frames, restrictions and precautions are considered to protect healing tissues and the surgical repair/reconstruction (Wilk, Macrina et al. 2010). General time frames are provided to reference the average rehabilitation time, but individual patients progress at different rates depending on their age, associated injuries, pre-injury health status, rehabilitation compliance and injury severity (Reinold, Wilk et al. 2006). A common rehabilitation protocol after microfracture surgery is known as continuous passive motion with protected weight bearing (6-8 hours/day for 6-8 weeks) (Tyler and Lung 2012, van Eekeren, Reilingh et al. 2012), during which the patients are required to stay in bed and the operation joint is moved by a mechanical device 8 hours per day (usually during sleep time). A few animal models showed that continuous passive motion can enhance the nutrition transport and metabolic activities of chondrocytes, stimulate chondrogenesis of MSCs, and accelerate the generation of cartilage (Salter, Bell et al. 1981, O'Driscoll, Keeley et al. 1988, Moran, Kim et al. 1992). However, despite its common use in practice, no strong scientific evidence is available to support the benefits of continuous passive motion. The ethical concern in clinical studies and high cost of animal models limited the search for optimized rehabilitation protocols, which may have countless combinations. Moreover, it is difficult to evaluate the outcome of microfracture in clinical practice, as current imaging technique is not precise enough to measure the tissue quality. Although there are some animal models for OA research, such as sheep and mini pig, it is difficult to guide the animals to follow specific rehabilitation protocols (Chu, Szczodry et al. 2010). A recent systematic review found only 4 valid studies about the effects of continuous passive motion in microfracture (Fazalare, Griesser et al. 2010). There is a great need for well-conducted, statistically valid studies to address the void in relevant literature.

Due to the lack of scientific evidence, the rehabilitation protocol after microfracture varies drastically between doctors. A recent survey in 2013 showed that over 50% of physical therapists apply partial weight bearing (PWB) directly, and the others wait for 1-8 weeks with passive motion or non-weight bearing (Figure 3.1) (Vogt, Angele et al. 2013). The starting time of weight bearing after microfracture surgery is purely empirical with no unified guidance. There is little information about the difference between direct loading and delayed loading. Steadman's group insists an 8-week long continuous passive motion generates the best outcome (Steadman, Briggs et al. 2003), but more and more surgeons apply partial weight bearing directly, which is also highly preferred by patients as they can walk with canes without 8-hours passive motion in bed.

Table 3.1 Parameters that need to be considered for rehabilitation after microfracture

Lesion	Patient	Surgery
Location	Age	Repair procedure
Size	Body mass index	Tissue involvement
Depth	General health	Concomitant procedures
Containment	Nutrition	
Quality of surrounding tissue	Quality of articular cartilage	
	Previous activity level	
	Specific goals	
	Motivation level	

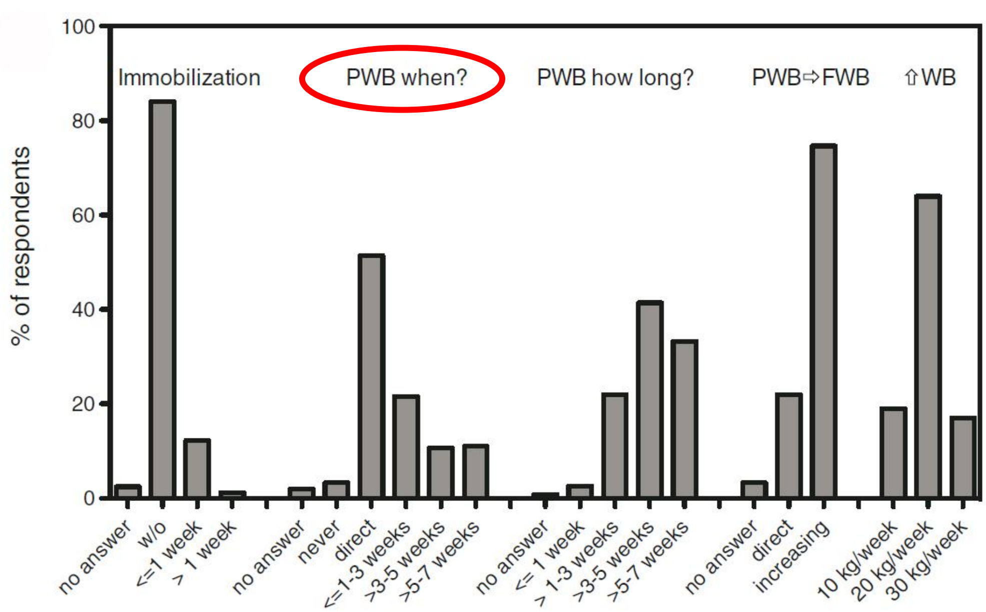

Figure 3.1 Comparison between immobilization, partial (PWB) and full weight-bearing (FWB) for start time and length of the treatment (Vogt, Angele et al. 2013).

It is well-known that dynamic compressive loading within appropriate ranges of magnitude and frequency can benefit the functional properties of articular cartilage (Mauck, Soltz et al. 2000, Mauck, Nicoll et al. 2003). Joint loading leads to complex tissue strains, including components of compression, tension, and shear, producing direct cellular and nuclear deformation (Guilak 1995). In addition, indirect biophysical factors are also generated as a result of the fluid exchange in between interstitial water and ions from cartilage, including streaming potentials, changes in local pH and osmolarity, and hydrostatic pressure (Guilak and Mow 2000). In cartilage tissue engineering research, it has been shown that mechanical stimulation can increase the synthesis of ECM such as proteoglycans and collagens (Sah, Kim et al. 1989, Larsson, Aspden et al. 1991, Steinmeyer, Ackermann et al. 1997), and this conclusion has been verified using various cell types, a variety of loading apparatuses and culture systems. Besides the direct physical stimulation on cells, mechanical loading are proved to be able to promote the nutrient transport significantly across the ECM or scaffold, which is critical for cell activities during *in vitro* culture (Bonassar, Grodzinsky et al. 2000). Many studies also proved that mechanical loading plays essential roles in the chondrogenesis of MSCs (Huang, Farrell et al. 2010). Moreover, some studies showed that prolonged unloading may result in weakening and atrophy of articular cartilage structure (Eckstein, Hudelmaier et al. 2006, Souza, Baum et al. 2012). During development, inhibition of muscle contraction and forces acting on skeletal elements results in abnormal joint formation (Mikic, Johnson et al. 2000, Mikic, Wong et al. 2000). Therefore, it is natural to postulate that mechanical loading could benefit the chondrogenesis of MSCs in the clot after microfracture, apply mechanical stimulations on the chondrocytes, and promote the nutrient transport and eventually the generation of new repaired tissue. An important unknown question is when the mechanical loading should start after the surgery.

In this study, using the *in vitro* microfracture system established in Chapter 2, we investigated the effects of mechanical loading and the start-time of mechanical loading on the formation of neocartilage at the lesion site.

3.2 Methods

In vitro microfracture models developed in Chapter 2 were employed to simulate the surgery. The samples were all cultured in Chondrogenic medium (DMEM with 10% FBS and 1% Insulin-Transferrin-Selenium (ITS), 0.1 μM dexamethasone, 1 mM sodium pyruvate supplemented with ascorbate acid (50 μg/ml) and 10 ng/ml of Transforming growth factor beta 3 (TGF-β3) for seven weeks. The medium was changed every other day. At week 2, 4, and 7, indentation tests were performed to evaluate the stiffness of new tissue, and the GAG and collagen contents were determined by biochemical assays. At the end of 7-week culture, the cell viability was examined with live/dead stain. Histology (H&E, Picro Sirius red and Safranin-O) and immunohistochemistry (type I and II collagens) analysis were performed. Expression levels of aggrecan, type I and type II collagen of cells at the lesion site were measured using qRT-PCR. Detailed protocols of the outcome measurements can be found in Chapter 2.

During the *in vitro* culture, dynamic compression was applied on the entire cartilage-bone explant every other day with a custom built loading device located in the biological safety hood (Figure 3.2). The explant was placed in between two parallel loading platens. A 20%-strain over the cartilage thickness was used as preload at a constant speed of 2 μm/s. After the response force from the sample relaxed to equilibrium value, sinusoidal dynamic loading was applied upon the static load at 0.5 Hz and ± 5% strain for 30 minutes each time. This mechanical stimulation profile is in the physiologic range, and has been widely adopted in cartilage tissue engineering studies (Hung, Mauck et al. 2004). During the loading, samples were submerged in culture medium supplemented with HEPES to buffer the PH value. Two different loading profiles simulating the actual rehabilitation protocols were utilized. One group samples had immediate mechanical stimulation from day 1 (Group 1: Direct) of culturing, and the other group had mechanical loading from day 15 (Group 2: Delayed). Both loading protocols were carried out until 7^{th} week of culture. An extra unloaded group of samples was cultured as control to identify the effects of mechanical stimulation.

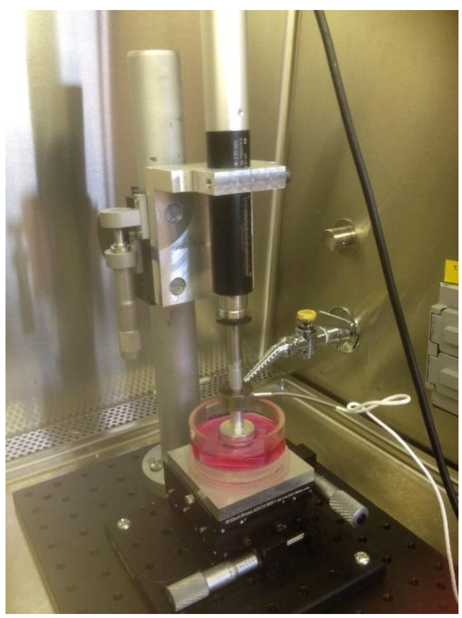

Figure 3.2 Mechanical loading device in the biological hood for dynamic compression stimulation.

A 2-way ANOVA with Bonferroni post-hoc analysis (or Scheirer-Ray-Hare test, if non-parametric) was used to compare the mechanical properties, GAG and collagen contents with effects for mechanical stimulation. Unpaired student's t-test (2-tail) was utilized to determine the significant difference between groups for the other parameters. All data are presented as mean ± standard deviation. Significance level was chosen at $p < 0.05$.

3.3 Results

3.3.1 Effects of Dynamic Loading

When compared to the unloaded group, mechanical stimulation in the loading group improved the cell viability in the surrounding cartilage and the subchondral bone at the end of 7-week culture (Figure 3.3). Although the cell densities are similar in the two groups, there are much less dead cells in the loaded group. Furthermore, more live cells were observed in the repaired new tissue at the lesion site in the loaded group, especially in the top layer. In the unloaded group, a large portion of cells in the repaired tissue are dead, but the top layer of new tissue in the loaded group has high-density live cells with few dead ones.

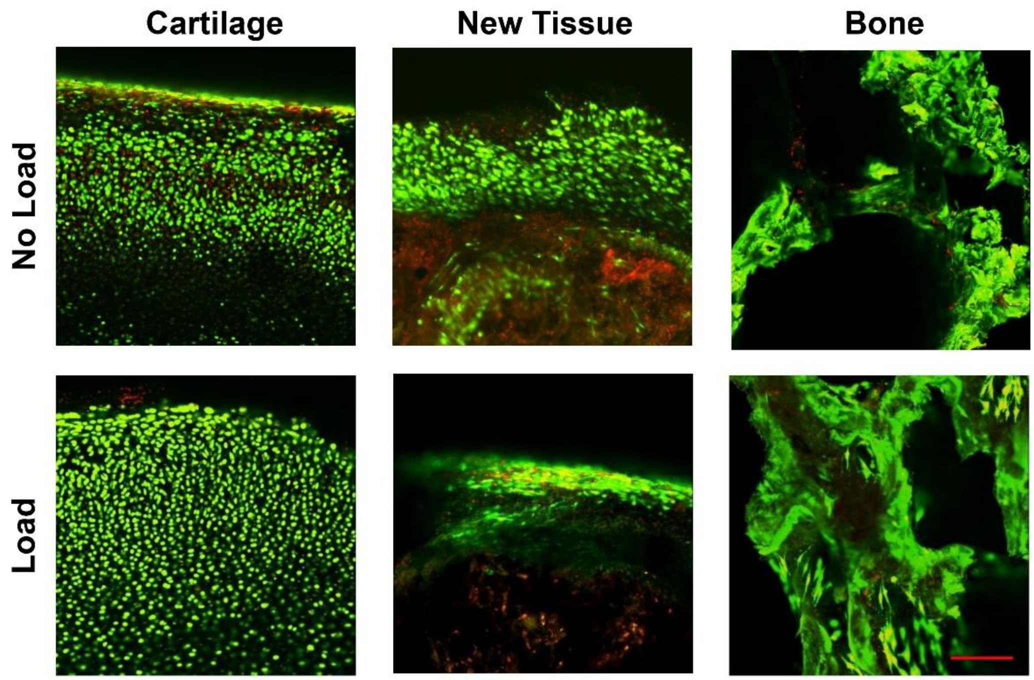

Figure 3.3 Cell viability by Live/Dead staining in native cartilage, repaired tissue, and subchondral bone for no load and loaded groups (Scale bar= 200 μm).

Collagen staining image showed that mechanical stimulation generated a dense layer of collagen fibers on the top of the lesion site, which is much thicker than that in the No Load group (Figure 3.4). All three histological staining images showed that more new tissues have been generated at the lesion site in the Load Group. The new tissue in the middle-deep zone almost filled the entire lesion, and the gap between the new tissue and subchondral bone vanished. The repaired cartilage is integrating with the subchondral bone.

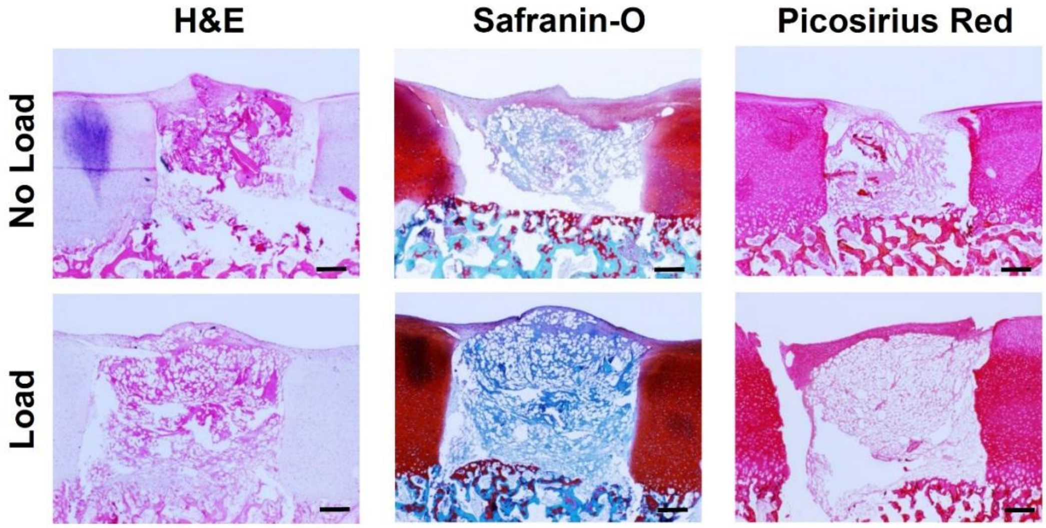

Figure 3.4 H&E, Safranin-O and Pico Sirius red staining on sample sections from No Load and Load groups (scale bar = 200 μm).

The GAG content in the newly generated tissue was significantly greater in the mechanically loaded samples than the unloaded ones at week 7, (4.29± 0.31 wt% vs 3.676± 0.59 wt%) (Figure 3.5A). Interestingly, no difference was detected in the instantaneous moduli between loaded and unloaded groups, except a significant difference at week 2 (no load: 1.83±0.58 kPa vs. load: 2.99± 0.39 kPa). The equilibrium modulus showed a similar trend. At week 2, the loaded group had much stiffer tissue than the unloaded samples (1.01±0.14 kPa vs. 0.52±0.11 kPa).

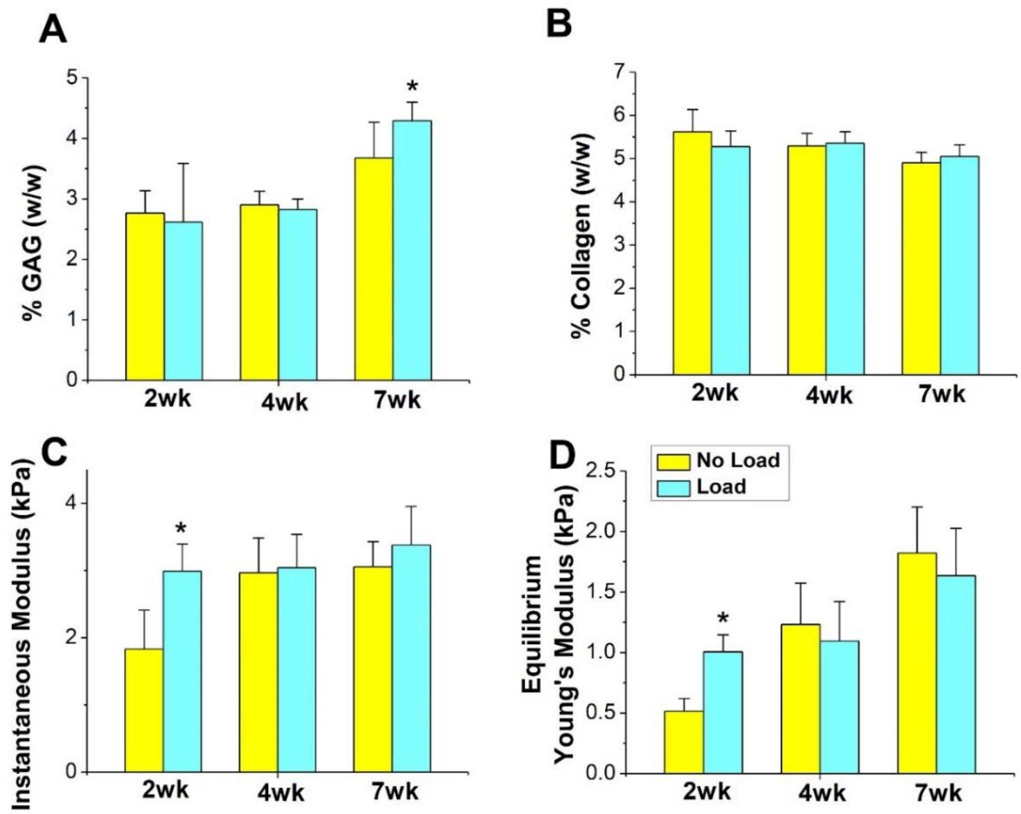

Figure 3.5 GAG (A) and Collagen (B) contents in the repaired tissue were compared between the loaded and unloaded groups. Mechanical properties were done by micro-indentation testing and Instantaneous (C) and Equilibrium Young's Modulus (D) were recorded (n=6; *: $p<0.05$, error bars: standard deviation).

In the unloaded group, little type I collagen was revealed by the immunohistochemistry, while the loaded samples has a thin layer of type I collagen on the top (Figure 3.6). No type I collagen was detected in the native cartilage after 7-week culture. In both unloaded and loaded groups, type II collagen was detected at the lesion site, with a bright thick band on the top zone and light stain across the entire thickness.

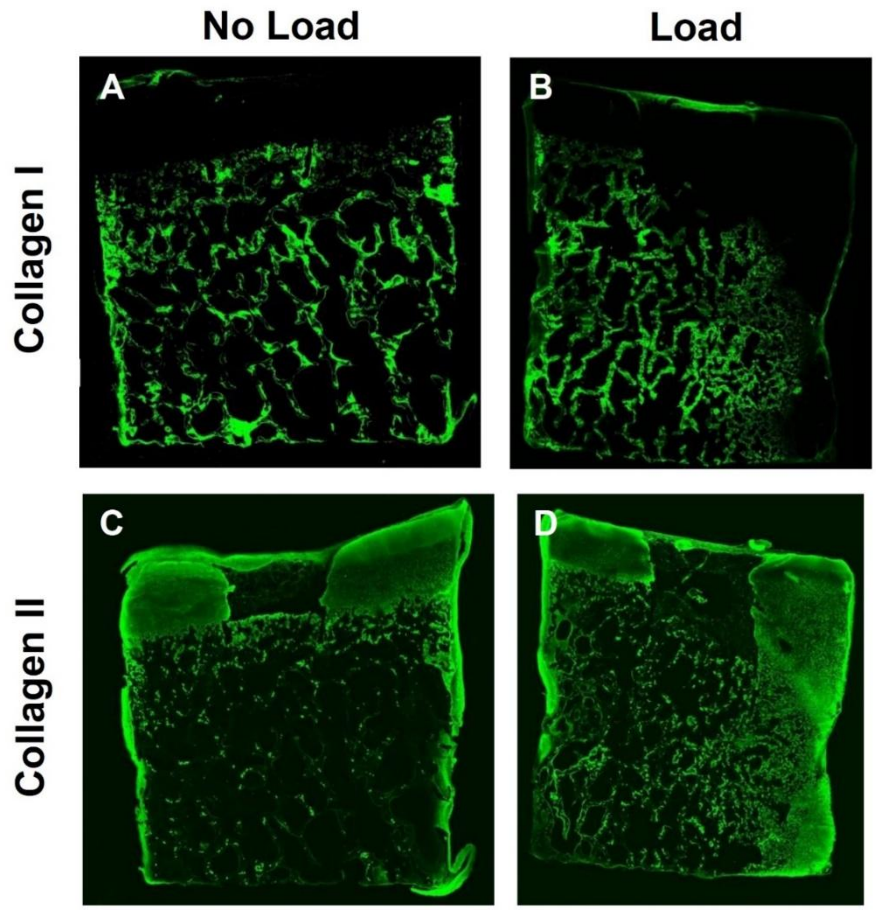

Figure 3.6 Immunohistochemical images with Collagen type I (A, B) and Collagen Type II (C, D) stainings on No Load and Load groups.

Expression of three chondrogenesis related genes was evaluated at the end of culture (7 weeks) and compared between no load and loading groups. Both type I collagen and aggrecan expression were significantly upregulated by the mechanical stimulation (Figure 3.7 A & C).

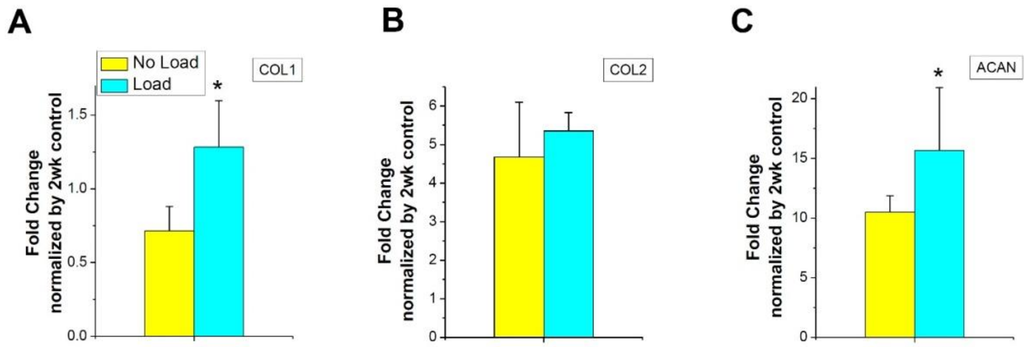

Figure 3.7 Gene expression levels after 7 week culture were evaluated with qRT-PCR. Fold change of Collagen type I (A), type II (B) and Aggrecan (C) were measured with GAPDH as the housekeeping gene (*: $p < 0.05$: n= 6 each, error bars: standard deviation).

3.3.2 Effects of delayed mechanical loading

Mechanical stimulation in both direct and delayed profiles improved the cell viability in the surrounding cartilage and bone when compared to the No Load group (Figure 3.8). Significantly more live cells present at the lesion site in both loaded groups, especially in the top layer of the repaired tissue. No significant difference was observed between the direct and delayed loading groups based on the live/dead staining images.

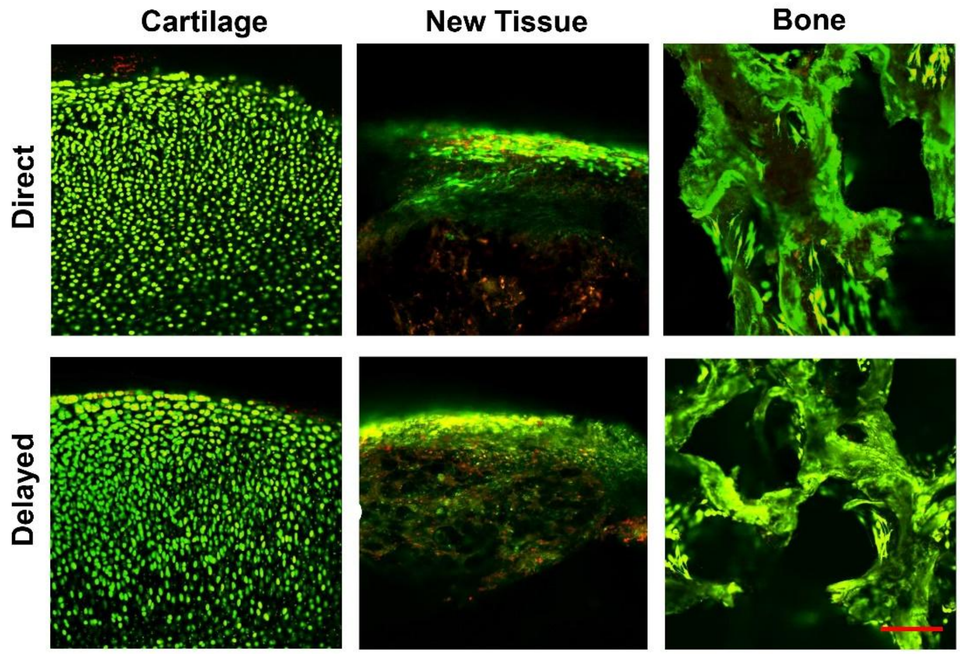

Figure 3.8 Live/Dead staining to visualize cell viability in cartilage, lesion site, and subchondral bone for Direct and Delayed groups (Scale bar= 200 µm).

Histology images showed that the lesion site of the delayed group is also fully filled with repaired tissue, similar to the direct load group (Figure 3.9). Again a dense layer of collagen fibers was formed on top of the lesion site. No significant difference was observed between direct and delayed groups in histological images.

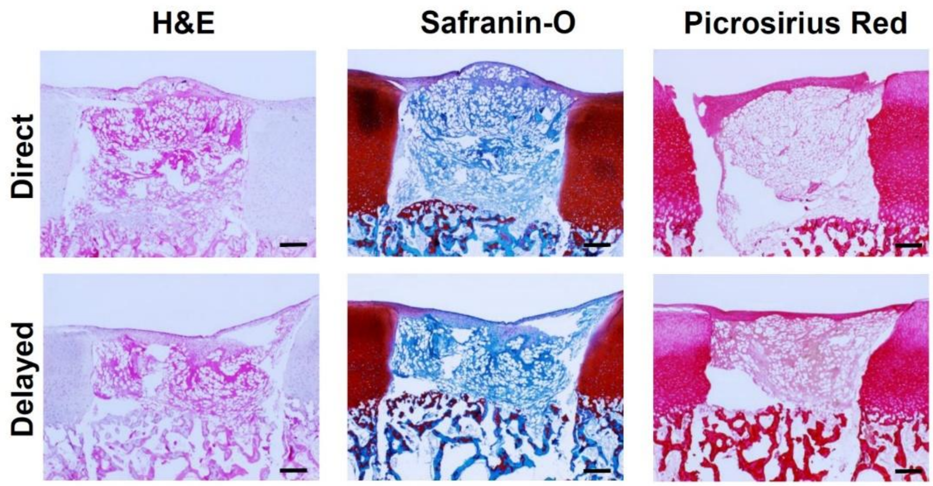

Figure 3.9 Histological stainings done on Direct and Delayed group (scale bar = 200 μm).

Biochemical assay showed that the GAG and collagen contents of the delayed group are significantly higher than the direct group. GAG content was 2.82 ± 0.17 % in the Direct group while the Delayed group was 3.8 ± 0.62 % at 4 week culture. After 7 weeks culture, GAG content increased to 4.29 ± 0.31 % in the direct group but 9.01 ± 0.8 % in the delayed group (Figure 3.10 A). Collagen content of the delayed group is also significantly higher after 7 week culture (Figure 3.10 B). Interestingly, the instantaneous mechanical properties at 2 week demonstrated the short-term benefit of mechanical stimulation (2.99± 0.4 kPa in the direct and 1.98 ± 0.57 kPa in the delayed group), but this trend was reversed at 7 week (3.93 ± 0.48 kPa) than the Direct (3.37 ± 0.57 kPa). Equilibrium Young's modulus showed a similar trend although no significant difference was detected at 7 week.

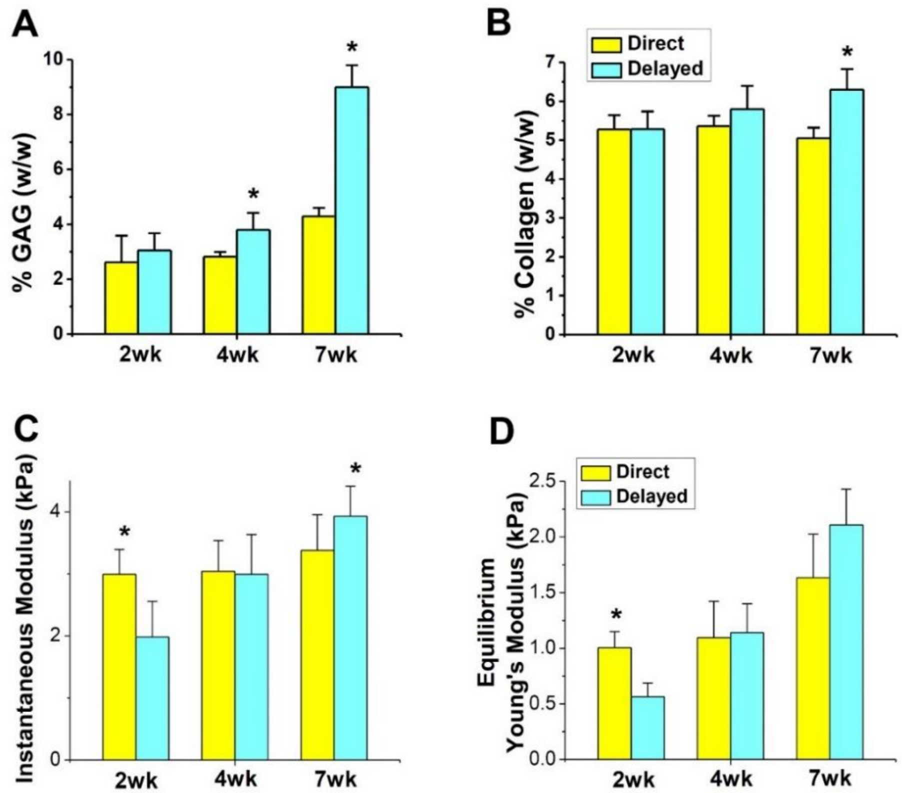

Figure 3.10 GAG (A) and Collagen (B) contents in the repaired tissue in direct and delayed groups. Instantaneous (C) and Equilibrium Young's Modulus (D) were measured by micro-indentation (n=6; *: $p<0.05$ error bars: standard deviation).

Immunostaining showed that both groups had a thick layer of Type I collagen on the top layer of repaired tissue. Type II collagen were detected at the lesion site in both groups (Figure 3.11). At the end of culture, significant upregulation of three genes, type I collagen, type II collagen, and aggrecan, were observed in the Delayed group (Figure 3.12). The fold change of the type I collagen to the 2 week control group came out as 1.283 ± 0.315 for the Direct and 2.726 ± 0.983 for the Delayed. The collagen type II gene expression was significantly higher than that of the unloaded samples and the fold change was 5.353 ± 0.472 for the Direct and 8.649 ± 1.857 for the Delayed. Similarly, Aggrecan expression level was significantly higher than that in the unloaded samples and the fold change was greater in the Delayed group than the Direct (15.66 ± 5.267 vs 21.34 ± 5.667).

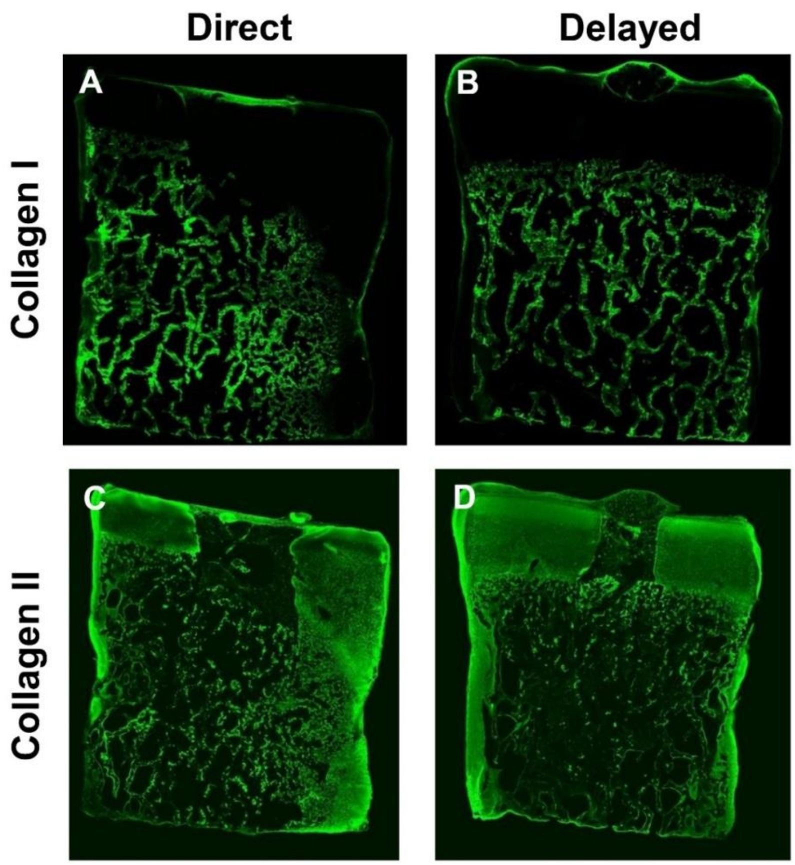

Figure 3.11 Immunohistochemical images on Direct and Delayed groups. Collagen type I (A, B) and Collagen Type II (C, D) stainings were performed.

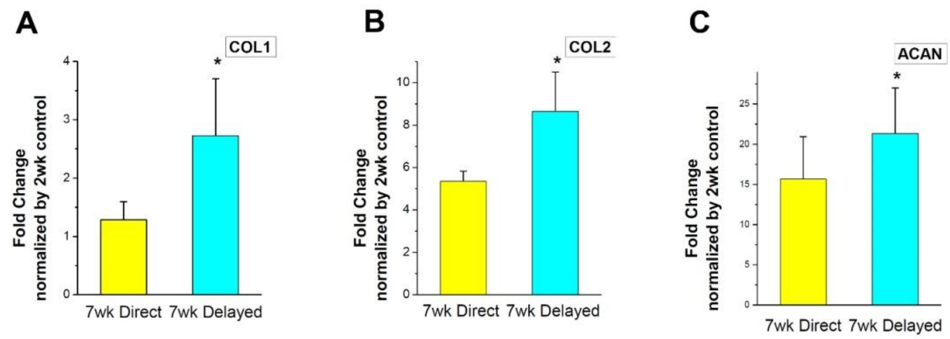

Figure 3.12 Gene expression results with 7 week cultured samples. Fold change of Collagen I (A), II (B) and Aggrecan (C) were measured (n=6; *: p<0.05).

3.4 Discussion

This study demonstrated that mechanical loading improved the chondrogensis of the cells at the lesion site of microfracture in comparison to free swelling culture. Dynamic loading significantly improved the cell viability of repaired tissue, tissue volume, GAG content and related gene expression. In cartilage tissue engineering studies, dynamic compression has proven its effectiveness in improving the functional properties of chondrocyte-seeded constructs (Mauck et al., 2000; Mauck et al., 2003a; Lima et al., 2006). Recent work with bone marrow MSCs showed that dynamic compression modulates the chondrogenesis of the cells (Kisiday, Frisbie et al. 2009, Li, Kupcsik et al. 2010, Thorpe, Buckley et al. 2010, Haugh, Meyer et al. 2011, Bian, Zhai et al. 2012). Mechanical loading on human or rabbit MSCs increased the expression of aggrecan and type II collagen (Angele, Schumann et al. 2004, Huang, Hagar et al. 2004) during 3D culture, and is mediated by induction of the TGF-β signaling pathway (Huang, Reuben et al. 2005). The presence of TGF-β also dictates the response of MSCs to compressive loading. Mechanical loading with the presence of TGF-β improved the proteoglycan synthesis of equine MSCs in comparison to free swelling culture (Kisiday, Frisbie et al. 2009). Although the present study cultured the bone marrow directly, the effects of mechanical loading are consistent with previous findings using isolated MSCs seeded in scaffolds. This implies that MSCs in the clot should be one of the major functional cells to generate new cartilage tissue. Another cell source for cartilage lesion repair is the migrating of chondrocytes from surrounding native cartilage (Mauck, Hung et al. 2003). However, in this study, no evidence demonstrated the migration of chondrocytes from native cartilage to the clot. It has been shown that migration of cells out of healthy cartilage could be difficult (Mauck, Byers et al. 2007), especially when the nutrient supply is very limited at the cartilage lesion site. Therefore, based on our *in vitro* study, the migration of chondrocytes from native cartilage is unlikely contributing to the generation of new cartilage.

Dynamic loading could benefit the outcome of microfracture using two mechanisms. First, the loading can provide various physical stimuli to the cells, including stress, strain, shear, hydrostatic pressure, and fluid flow. These physical signals have been proven to be critical for the chondrogenesis of chondrocytes (Estes, Gimble et al. 2004, Wescoe, Schugar et al. 2008, Grad, Eglin et al. 2011). Moreover, the mechanical loading can also significantly benefit the native cartilage and subchondral bone during *in vitro* culture (Chan, Lu et al. 2009, Hattori, Uematsu et al. 2009, de Vries-van Melle, Mandl et al. 2012). Improved anabolic activities from surrounding cartilage and bone could further benefit the chondrogenesis of MSCs in the clot. Second, dynamic loading can significantly improve the nutrient transport (Albro, Chahine et al. 2008, Chahine, Albro et al. 2009). Since the major component of bone marrow is fat and the blood clot is not porous, the transport of nutrients to the middle-deep layer could be extremely challenging. This has been proved with the results of free-swelling culture. The top layer had much more new cartilaginous tissue as the cells on the surface can access the culture medium. In contrast, the middle-deep zone had much less new tissue. When the explants are under dynamic loading, medium could be pumped in and out of the lesion site. The fluid phase inside the native cartilage could also be squeezed into the lesion site. The moving of fluid phase could bring nutrients into the lesion site and take away the metabolic wastes. Both mechanisms could contribute to the growth of new tissue in the middle-deep zone. In this study, mechanical loading did not significantly increase the stiffness of new tissue. Mechanical properties from micro-indentation are mainly affected by the tissue on the top surface. The top layer tissue in the unloaded group is similar to that in the loaded group. Thus the mechanical properties are similar. However, due to the promoted nutrient transport across the middle-deep zone, the tissue volume was significantly increased and chondrogenic gene expression was upregulated. Interestingly, type I collagen, besides the type II fibrils, was noticed on top of the new tissue and surrounding cartilage in the loaded explants. Therefore, under mechanical stimulation and with TGF-β3 supply, the progenitor cells in the superficial zone or bone marrow showed fibroblast phenotype. In previous studies, this fibrous "capsule" has been always noticed in neocartilage generated by MSCs during in vitro culture, especially when the cell-seeded scaffold was mechanically stimulated (Bueno, Bilgen et al. 2005).

Although there is no doubt that the mechanical loading can benefit the chondrogensis of chondrocytes during *in vitro* culture, the starting time of loading could affect the results. A recent study showed that delayed mechanical loading on

MSCs seeded in agarose gel generated stiffer cartilage construct than mechanical loading started immediately after cell seeding (Huang, Farrell et al. 2010). In another study, dynamic compression (initiated immediately after porcine MSC encapsulation in agarose) was carried out over 42 days in the presence of TGF-β. A marked reduction in the mechanical properties of loaded constructs was detected in comparison to free-swelling controls (Thorpe, Buckley et al. 2008). It was now concluded that loading initiated after chondrogenesis and matrix elaboration can further improve the mechanical properties of MSC-based constructs, but it was unclear whether the same mechanism exists in the bone marrow stimulated cartilage lesion repair. The present study, for the first time, demonstrated that a delayed mechanical loading after microfracture may benefit the outcome when compared to immediate loading. MSCs in the bone marrow clot require certain time to differentiate and to form ECM surrounding them. Immediate loading may have adverse effects during this period. Therefore, as the success of microfracture relies on the formation of a blood clot at the site of the defect, protection of the clot is essential during this early time frame. This result may have significant implications for the design of new postoperative rehabilitation protocols and provide guidance for future *in vivo* studies.

It is important to note that movement of joint after microfracture is not only designed to apply mechanical loading at the lesion site. It is also critical for the health of entire joint, such as native cartilage, ligament, tendon, bone and muscles. Animal models of reduced loading report that a decrease in mechanical stimuli leads to atrophy of the tissue (Jurvelin, Kiviranta et al. 1986, Haapala, Arokoski et al. 1999, Haapala, Arokoski et al. 2000), and ultimately erosion of the articular cartilage (Hagiwara, Ando et al. 2009). In humans, reduced loading in the form of partial weight bearing for 7 weeks results in significant cartilage thinning in the knee articular cartilage (Hinterwimmer, Krammer et al. 2004). Thus the use of a continuous passive motion device (CPM) as a tool to assist nourishing the surrounding articular cartilage is commonly advocated. However, it could be beneficial to avoid direct loading on the lesion site during the first two weeks.

3.5 Conclusions

Bone marrow filled cartilage-bone explants were cultured long term *in vitro*, which simulated the tissue regeneration progress after microfracture surgery. Mechanical stimulation significantly improved the biochemical and mechanical properties of the newly generated tissue at the lesion site. More importantly, the 2-week delayed loading demonstrated more beneficial effects on the regenerated tissue's quality compared to the immediate loading. It implies that a delayed partial weight bearing on the lesion site in rehabilitation protocol could be more beneficial than the immediate loading.

Chapter 4

ZOLEDRONIC ACID (ZA) FOR THE PREVENTION OF PTOA

4.1 Introduction

Osteoarthritis (OA) is affecting millions of people in the United States, and could result from systemic factors (such as genetics, dietary intake, estrogen use, and bone density) and from local biomechanical factors (such as muscle weakness, obesity, and joint laxity) (Felson, Lawrence et al. 2000). Post-traumatic osteoarthritis (PTOA) is an accelerated development of OA after joint trauma, e.g., meniscal or ligament tears. It is important to notice that the traumatic damage is not necessary on cartilage itself. In many cases, trauma damages other soft tissues in joint. For example, trauma could break ACL or meniscus in the knee joints, leading to cartilage degeneration later. During PTOA progression, the apoptosis and alteration of chondrocyte metabolism may play a critical role (Kramer, Hendricks et al. 2011).

Surgical strategies to restore joint functions after traumatic injuries, such as repairing ligament and meniscal ruptures, have been well developed. However, the surgery cannot prevent the development of long-term PTOA in more than 40% of patients (Anderson, Chubinskaya et al. 2011). Indeed few medical therapies are currently available to prevent the initiation of OA after joint injuries. Due to the challenge in the early detection of OA, PTOA usually presents as moderate-to-severe degeneration at the time of diagnosis, restricting interventions to late-stage therapies similar to those used for idiopathic OA, such as microfracture, autologous cartilage transplantation, and Autologous chondrocyte implantation (ACI). Ultimately PTOA can lead to total loss of joint function, requiring joint arthroplasty to provide symptomatic relief. However, PTOA is different with primary OA because it is expected in a certain time period. This provides a unique time window to prevent or postpone the initiation of cartilage degeneration.

Zoledronic acid (ZA, Molecular Weight: 290) is an FDA approved bisphosphonate (BP) for the treatment of resorption-related bone loss. BPs preferentially binds to calcium and the largest store of calcium is in bones. When osteoclasts are removing the bone, the drug can get ingested into the cells and disturb the cytoskeleton metabolism. A chondro-protective effect following bisphosphonate (BP) treatment in PTOA was noticed more than a decade ago. Podworny et al. found that subcutaneous injections of ZA partially protected articular cartilage from inflammatory arthritis induced by carrageenan, without affecting the induced synovitis (Podworny, Kandel et al. 1999). Later the chondro-protective capability of alendronate was revealed in a rat ACL transection model (Hayami, Pickarski et al. 2004). Two recent studies using different PTOA animal models (rabbit osteochondral defect and rat monosodium iodoacetate injection) confirmed these earlier findings (Nishitani, Shirai et al. 2009, Strassle, Mark et al. 2010). Our preliminary studies investigating cartilage degeneration in the destabilized medial meniscus (DMM) mouse model of PTOA found the repeated systemic administration of ZA to be highly successful in preventing cartilage damage (Figure 4.1). It is critical to note that in all these studies, OA was induced by well-defined traumatic injuries and the administration of BPs occurred immediately after the trauma. Inspired by such promising animal results, a large randomized trial in humans was conducted in both North America and the EU (Bingham, Buckland-Wright et al. 2006). Enrolled patients suffered from knee pain, knee stiffness, or knee crepitus and were diagnosed with narrowed joint space. However, little positive effect of risedronate treatment on idiopathic OA was demonstrated in this large study. Based on these data it was suggested that the timing of bisphosphonate treatment could be critical to OA (Saag 2008). This idea has been reinforced by multiple clinical late-stage OA trials with BPs that have shown equivocal effectiveness (Carbone, Nevitt et al. 2004, Bingham, Buckland-Wright et al. 2006), whereas results from animal model studies continue to prove the capacity of BPs to inhibit the initiation of OA. Thus, the initiation and progression of OA may involve different pathogenesis, and could require distinct treatment strategies (Buckwalter, Mankin et al. 2005, Kurz, Lemke et al. 2005, Chaudhari, Briant et al. 2008, Szczodry, Coyle et al. 2009, Anderson, Chubinskaya et al. 2011). Literature searches provide no insight into the cellular or biochemical mechanisms of BP mediated chondro-protection in PTOA pathogenesis.

Using the DMM (destabilization of medial meniscus) mouse model, our preliminary study found that systemic injection of ZA could suppress the development of PTOA. As shown in Figure 4.1, after two months, there was osteoarthritis with significant cartilage degeneration in the control group. In the ZA treated group, the joint had very minor damage sign on cartilage. The average OA damage scores were significantly higher in the non-treated group than the ZA treated animals for both tibia and femur sides. This preliminary data, as well as several published studies, have demonstrated an encouraging chondro-protective capability of BPs, which if properly leveraged could lead to a revolution in PTOA treatment. However, no studies have been performed to identify the chondro-protective mechanisms of BPs at the cellular or molecular levels. In earlier OA studies, it was conjectured that the chondro-protective effect of BPs resulted from their inhibition on osteoclast activities and bone resorption (Podworny, Kandel et al. 1999, Hayami, Pickarski et al. 2004, Bingham, Buckland-Wright et al. 2006, Nishitani, Shirai et al. 2009, Strassle, Mark et al. 2010), yet no scientific evidence is available to directly support this hypothesis. Furthermore, if this hypothesis holds true it would significantly limit the application of BPs in the treatment of PTOA. This is because most joint trauma patients may not have osteoporosis and, systematic use of BPs in "bone-healthy" young adults may interfere with regular bone remodeling and have adverse long-term skeletal effects. However, it has been noticed that BPs can affect the fate of chondrocytes in the absence of osteoclasts (Van Offel, Schuerwegh et al. 2002, Dombrecht, Schuerwegh et al. 2007, Evans and Oberbauer 2009) and that the inhibition of the mevalonate pathway has direct protective effects in cultured osteoarthritic chondrocytes (Simopoulou, Malizos et al. 2010).

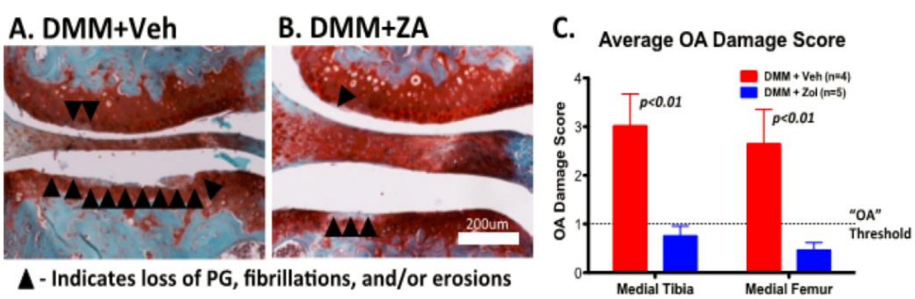

Figure 4.1 Continuous administration of zoledronic acid (ZA) to treat bone loss, suppressed the development of post-traumatic OA in a DMM mouse model (destabilization of the medial meniscus). (A, B) Histological analysis and (C) Cartilage damage scoring of DMM and ZA treated mouse knees (Veh: Vehicle control group) (Price C. et. al., ORS Annu Conf, 2011).

In our previous two chapters, it has been demonstrated that cartilage-bone explants can be culture for long-term. *In vitro* cartilage culture has been widely adopted over the last two decades as a powerful investigational tool (Patwari, Gaschen et al. 2004, Lee, Fitzgerald et al. 2005, Bian, Lima et al. 2008). Cartilage explants can be cultured for months given the proper nutrient and oxygen supplies. In culture, the extracellular matrix can be dramatically remodeled by the embedded chondrocytes, and it has been shown to be controlled by various physical or cytokine stimulations (Hunziker 2002, Mauck, Nicoll et al. 2003). If the presence of BPs in culture medium could prevent or reduce the chondrocyte apoptosis in trauma loaded cartilage explants, it would represent convincing evidence to support our hypothesis. A distinct advantage of the *in vitro* model is the convenience of longitudinal evaluation of cartilage biomechanical properties, which is a technical challenge *in vivo* (Buckwalter, Mankin et al. 2005, Brown, Johnston et al. 2006). Mechanical testing can be performed across multiple time points in a sterile environment without compromising the long-term culture. Therefore we will use cartilage explants to investigate the effect of BPs on chondrocytes.

Intracellular calcium signaling is one of the earliest responses in chondrocytes under physical stimuli (Kono, Nishikori et al. 2006, O'Conor, Leddy et al. 2014, Sanchez-Adams, Leddy et al. 2014). It is an essential regulator of the mechanotransduction process as well as the upstream of many signaling pathways in chondrocytes. It is involved in the regulation of various physiological processes such as secretion and gene expression (Grandolfo, Calabrese et al. 1998, Pritchard and Guilak 2006, Sanchez-Adams, Leddy et al. 2014). Chondrocytes are isolated in cartilage and the calcium wave propagation is facilitated by the diffusion of messengers, which is an essential intercellular communication pathway in cartilage (Kono, Nishikori et al. 2006). Also, both isolated and *in situ* chondrocytes have been found to be able to release spontaneous $[Ca^{2+}]_i$ signaling (Kono, Nishikori et al. 2006, Fodor, Matta et al. 2013, O'Conor, Leddy et al. 2014), *i.e.*, the $[Ca^{2+}]_i$ concentration oscillates in chondrocytes without the presence of any extraneous mechanical or chemical stimuli. Due to the critical role of $[Ca^{2+}]_i$ signaling in chondrocyte mechanotransduction and cartilage remodeling, we further hypothesize that the beneficial mechanisms of ZA supplemented culture of cartilage explants is associated with the spontaneous $[Ca^{2+}]_i$ signaling in chondrocytes.

In this chapter, we tested the hypothesis that zoledronic acid can directly regulate the metabolic activities of chondrocytes. Two specific aims are performed. First, using cartilage explant *in vitro* culture model, we studied whether supplement of ZA can promote the beneficial effects of mechanical stimulation. The second aim is to study whether the chondro-protective effects of ZA is correlated with the spontaneous calcium signaling of *in situ* chondrocytes.

4.2 Methods

4.2.1 Cartilage Explant Culture

Cylindrical cartilage explants were harvested from the femoral condyle head of 3~6 month-old fresh calf knee joints (Green village, NJ) using a 3 mm biopsy punch. Superficial-to-middle zone of the cartilage (2 mm in thickness) was obtained with a custom designed cutting tool. The full thickness of cartilage ranges from 4mm to 6mm. After harvesting, cartilage explants were cultured in chondrogenic medium (DMEM, 1% ITS+Premix, 50 μg/ml L-proline, 0.1 μM dexamethasone, 0.9 mM sodium pyruvate, 50 µg/ml ascorbate 2-phosphate) with or without 1 µM ZA (Zometa®, Novartis) for 4 weeks. Due to the small molecular weight of ZA, it should be able to easily diffuse across the 3 mm cartilage explant. The culture condition is 37 °C and 100% humidity with CO_2 level (5%). Culture medium was changed every other day. During the culture, samples were stimulated with mechanical loading every day. A 10% preload was applied on the tissue at a speed of 2 µm/s. After the response force reaches equilibrium level, dynamic loading of the ±3% strain of the height of the cartilage was applied at 0.5 Hz for 30 min daily.

4.2.2 Longitudinal Mechanical Testing

Mechanical properties of cartilage explants, including equilibrium Young's modulus and dynamic modulus, were measured using unconfined compression test on day 1, 1wk, 2wk and 4wk using a custom built loading device. The original thickness of cartilage sample was measured as the distance between upper and lower platens with 5-gram contact force. A 10% strain was applied on the tissue at a constant speed of 2 µm/s followed by 10 minutes relaxation period. After the force reaches the equilibrium state, sinusoidal dynamic loading was applied at 0.5 Hz and ± 1% strain. Equilibrium Young's modulus and dynamic modulus of the sample were determined from the recorded force history during test (Lu and Mow 2008).

4.2.3 Spontaneous $[Ca^{2+}]_i$ Signaling

On days 2, 8, and 15, five extra cartilage explants were cut into two identical halves to record the spontaneous calcium signaling of *in situ* chondrocytes. Cartilage explants were cut into two identical halves using a cutting tool and loaded with 5 µM Fluo-8 AM (AAT Bioquest, CA) for 40 min. After fluorescent dye, samples were washed in pure DMEM three times, for 5 min each time. The half cylindrical sample, with cross-section area facing down in an imaging chamber, was mounted on a confocal microscope (Zeiss LSM510) (Figure 4.2). The objective lens was focused at the center region close to the superficial zone of cartilage. Fluorescent images of chondrocytes were recorded every 1.5 seconds for 15 minutes. Intracellular calcium responses of individual cells were analyzed by image processing technique described before (Huo, Lu et al. 2008, Huo, Lu et al. 2010, Lu, Huo et al. 2012). $[Ca^{2+}]_i$ curve was obtained by measuring the average image intensity of each cell region. A cell was defined as responsive if it showed a calcium peak with a magnitude four times higher than its fluctuations along the baseline (Donahue, Donahue et al. 2003, Donahue, Haut et al. 2003). The responsive rate is defined as the ratio of responsive cell number to the total number of cells in the fluorescent image. The number of $[Ca^{2+}]_i$ peaks during 15-minutes period were counted for all the responsive cells. To further analyze the calcium response curve of individual cells, spatiotemporal properties of the $[Ca^{2+}]_i$ peaks were extracted as shown in Figure 4.3. The time between the starting point from the baseline and the maximum value of a $[Ca^{2+}]_i$ peak was defined as "time to reach a peak"; The time from a $[Ca^{2+}]_i$ peak dropping to its 50% was defined as the "peak relaxation time"; And the time interval between two successive $[Ca^{2+}]_i$ peaks was defined as "time between two peaks" (Lu, Huo et al. 2012).

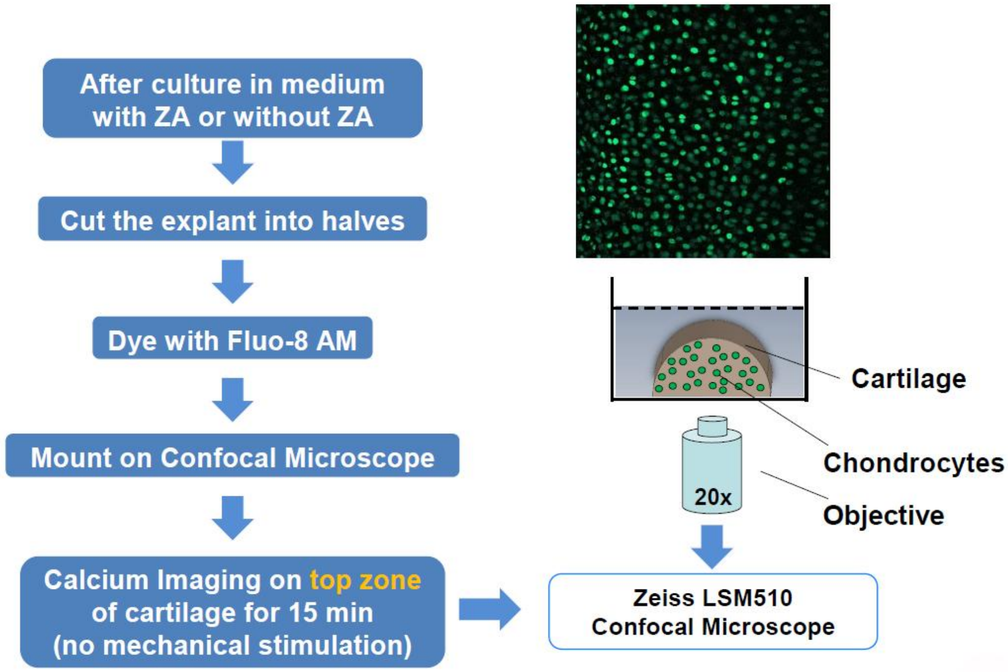

Figure 4.2 Illustration of calcium imaging for in situ chondrocytes in a cartilage explant. Half cylindrical cartilage explant in a glass-slide imaging chamber is mounted on a confocal microscope.

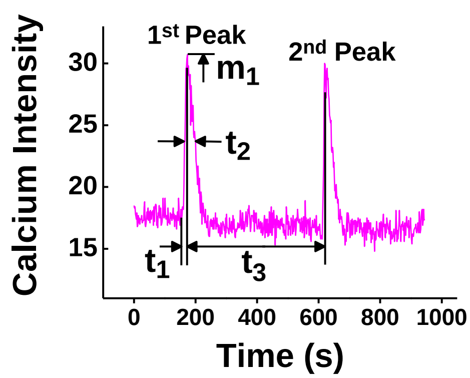

Figure 4.3 A typical $[Ca^{2+}]_i$ intensity oscillation curve of a chondrocyte over 15 minutes and the definition. The $[Ca^{2+}]_i$ intensity was represented by average image intensity inside a normalized cell over baseline level. Number of $[Ca^{2+}]_i$ peaks, magnitude of 1st peak m1, time to 1st peak t1, relaxation time of 1st peak t2, and time interval between two neighboring peaks t3 were also defined here.

4.2.4 Gene Expression

Expression level of aggrecan (ACAN), type I collagen (COL1), type II collagen (COL2), ADAMTS5, and MMP-13 genes were tested after 2 weeks of culture (n=6). The sequences of the primers for ACAN, COL1 and COL2 were listed in Table 2.2. The made-to-order genes proprietary to Life Technologies were utilized for ADAMTS5 (Product.# Bt04230789_m1), MMP13 (McGovern, Carson et al. Bt03214052_m1) and GAPDH (McGovern, Carson et al. Bt03210912_g1) and the sequences are not disclosed. The relative gene expression was calculated using the $\Delta\Delta C_t$ method, where fold difference was calculated using the expression $2^{\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Each sample was internally normalized to the reference gene, GAPDH, and each group was normalized to the expression levels of the control. Relative expression levels that are greater than 1 represent upregulation with ZA treatment, while relative expression levels less than 1 represent downregulation of the gene treated with ZA compared to the control.

$$\Delta\Delta \mathrm{C}_T = \left(C_T(\text{target, untreated}) - \mathrm{C}_T(ref, untreated)\right) - (C_T(target, treated) - C_T(ref, treated))$$

4.2.5 Statistical analysis

Student's t-test for unpaired data (2-tail) was utilized to determine significant difference between the control and treated groups. All data is presented as mean ± standard deviation. The measured variables are correlated to culture time using linear regression. Statistical significance was established at $p < 0.05$.

4.3 Results

4.3.1 ZA Enhanced the Beneficial Effects of Mechanical Stimulation

Mechanical stimulation was applied along with the ZA treatment during the 4-week culture of cartilage explants. When compared to the initial values on D3, Young's modulus increased 110% in the control group and 172% in the ZA group treated group after 4 weeks culture (Figure 4.4). Significant difference was detected between the two groups at 2 weeks culture, while the Young's moduli were 0.69±0.19 MPa vs. 1.04±0.43 MPa for control and ZA group, respectively. After 4 weeks culture with mechanical stimulation, the Young's moduli increased to 1.14±0.27 MPa for control group and 1.63±0.26 MPa for ZA supplemented group. Dynamic modulus showed a similar trend with the Young's modulus, although the gap between two groups was smaller. It is clear that supplement of ZA increased the mechanical properties of cartilage explants after 2 weeks *in vitro* culture.

RT-PCR showed that the expression of type I collagen in ZA group was 3.3 times higher than the control group. Expressions of both type II collagen and aggrecan were significantly upregulated in the ZA treated group. Expression levels of ADAMTS5 and MMP13, two of the most critical catabolic genes, were significantly downregulated in the ZA group in comparison to the control group (Figure 4.5).

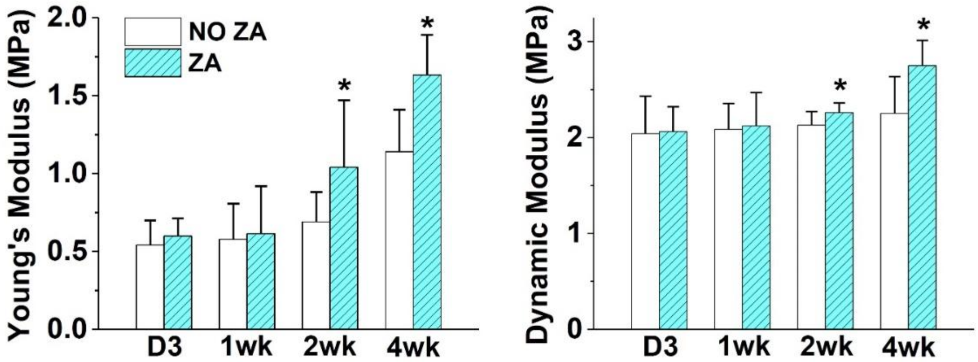

Figure 4.4 Young's modulus and dynamic modulus of cartilage explants cultured with daily mechanical stimulation in medium supplemented with or without ZA (n=6; *: p<0.05, error bars:standard deviation).

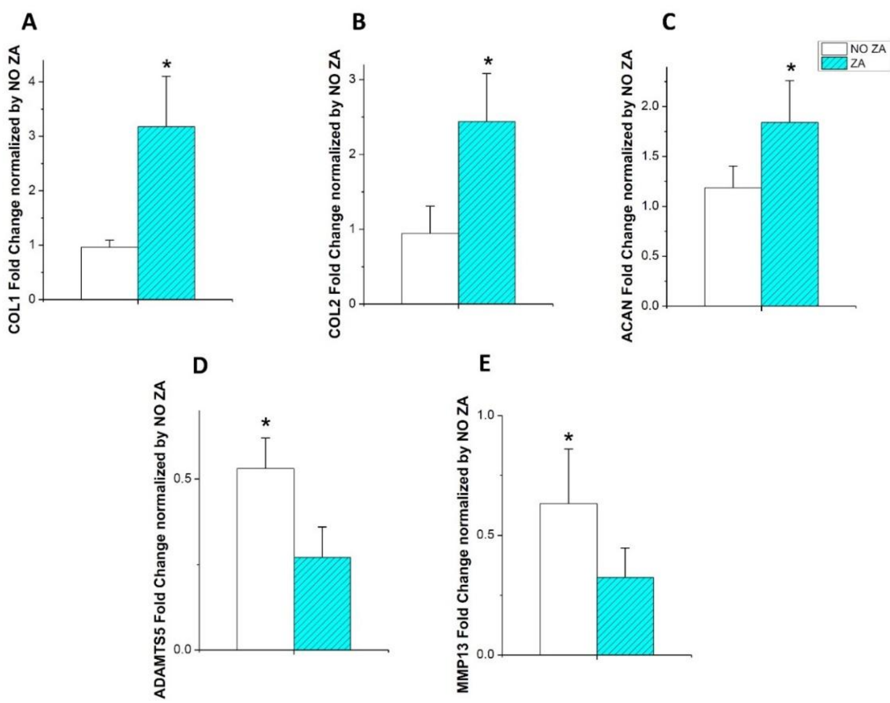

Figure 4.5 Gene expression levels of chondrocytes measured by RT-PCR after 2 week culture with mechanical loading. (A) Collagen I, (B) Collagen II, (C) Aggrecan, (D) ADAMTS5 and (E) MMP13 (n=6; *: p<0.05, error bars:standard deviation).

4.3.2 Spontaneous $[Ca^{2+}]_i$ Signaling and ZA Treatment

Spontaneous calcium signaling of *in situ* chondrocytes was recorded for cartilage samples in both groups on days 2, 8 and 15 during free swelling culture (no daily mechanical stimulation). Spontaneous responsive rates were significantly different between two groups. The responsive rate stayed constant at ~20% in the control group during the culture, while the responsive rate increased steadily from 32.2% at day 2 to 39.5% at day 8 and 45.4% at day 15 in ZA treated group (Figure 4.6 A). Average number of calcium peaks in the responsive cells showed differences between two groups at all time points. On day 2, the numbers of peaks were 1.87±1.29 vs. 1.67±0.87 for No ZA and ZA, respectively. However, this trend was reversed, and samples treated with ZA showed significantly more calcium peaks than the control group at days 8 and 15. The peak relaxation time was higher in ZA group than the control group at day 2, but at day 15, 59.5±23.6 s was required for the cells to relax in the control group and only 42.6±27.9 s for the ZA group. Magnitude of calcium peaks in ZA group was significantly higher than the control group at all-time points. Time between two neighboring calcium peaks was significantly longer in ZA treated group at day 2 (ZA: 319±140.7s vs Control: 223.2±155.2 s). Again, the trend was reversed at day 15 (ZA: 180.2±141.3 s vs Control: 239.7±157.6 s). Time to reach a calcium peak was significantly longer in the ZA group at days 2 and 15 than the corresponding control group.

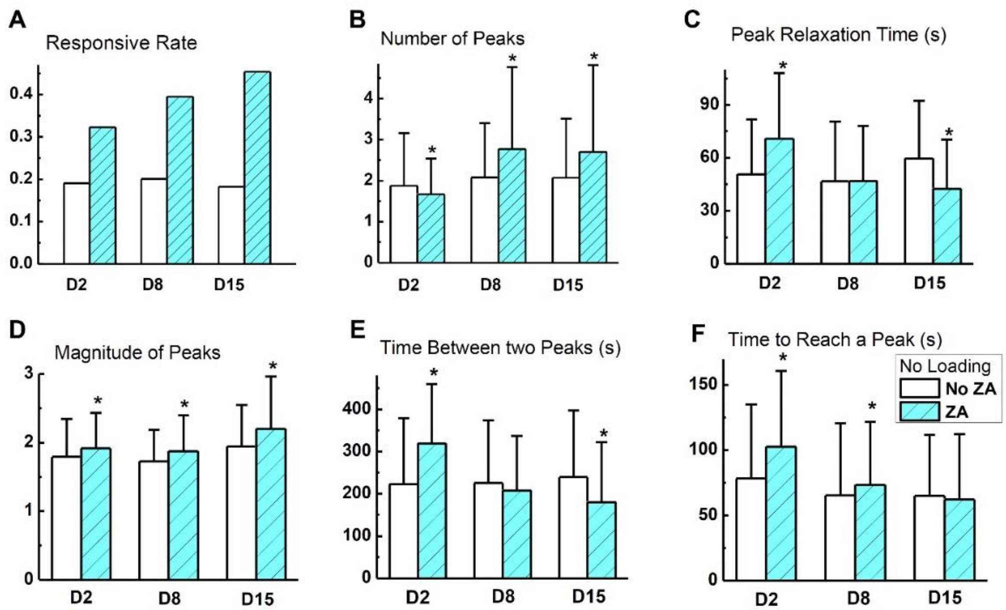

Figure 4.6 Calcium signaling of *in situ* chondrocytes in cartilage explants cultured with or without ZA. (A) Responsive percentage of cells; (B) Number of calcium peaks of responsive cells; (C) The time for a peak to relax to 50% of its peak magnitude; (D) Average magnitude of $[Ca^{2+}]_i$ peaks; (E) Time between two neighboring peaks; (F) Average time to reach a calcium peak from baseline (n> 160 cells; *:p<0.05, error bars:standard deviation).

Calcium signaling were also recorded and compared for the cartilage explants that received daily mechanical stimulation during the culture. The responsive rate of chondrocytes responses showed a similar trend to the free swelling culture samples summarized in Figure 4.6. The responsive rate of the No ZA group was maintained at ~20% while the ZA group was significantly higher with 32.4% at day 2, 35.8% at day 8 and 38.8% at day 15. More calcium peaks were reported at days 8 and 15 in ZA treated group than the corresponding No ZA group (Figure 4.7B). Relaxation time of calcium peaks was shorter in ZA group than the control group. At day 2, it was reported as 30.4±19.6 s versus the 47.3±17.0 s for the No ZA group. At day 8, the relaxation time of ZA groups was 23.5±10.7 s versus the 34.9±14.7 s for No ZA group (Figure 4.7C). Magnitude of calcium peaks showed no significant differences between two groups. Time interval between two neighboring peaks was generally lower in the ZA group, but the difference is only significant at day 15. Time to reach a peak in the ZA group is significantly lower at day 2 than the control (Control: 42.2±14.2 s vs. ZA: 24.3±18.4 s) (Figure 4.7F).

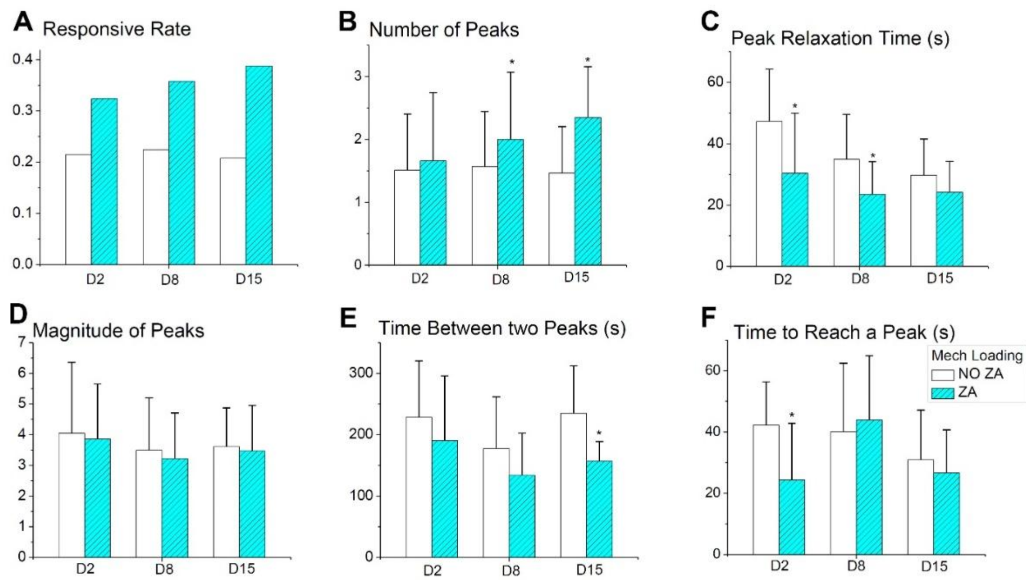

Figure 4.7 Spontaneous $[Ca^{2+}]_i$ signaling of chondrocytes in cartilage explants with daily mechanical stimulation. (A) Responsive rate of chondrocytes; (B) Number of calcium peaks on samples that responded; (C) Peak relaxation time; (D) Average magnitude of $[Ca^{2+}]_i$ peaks; (E) Time between two neighboring peaks; (F) Average time to reach a peak from baseline (n >160 cells; *:p<0.05, error bars:standard deviation).

4.4 Discussion

Bisphosphonates are commonly used as anti-osteoporotic drugs, exerting both mineralization inhibition and osteoclast apoptotic effects that make them well suited to inhibit bone resorption (Fleisch, Russell et al. 1966, Fleisch, Russell et al. 1969). From the physiochemical side, bisphosphonates could inhibit the formation of the ruffled border of the osteoclast surface (Sato and Grasser 1990), which facilitate osteoclasts to detach from the bone. This process results from the inhibition of mevalonate pathway of osteoclasts by bisphosphonates. To be specific, they can block protein geranylgeranylation, leading to malfunction of the osteoclast cytoskeleton (Coxon, Helfrich et al. 2000). Recently, these chemicals have also been noticed to have anti-inflammatory, chondro-protective, and anti-catabolic effects, and were considered as the potential drugs to treat osteoarthritis and rheumatoid arthritis (Van Offel, Dombrecht et al. 2005, Dombrecht, Schuerwegh et al. 2007). Several studies on osteoarthritis in animals provide a rationale for the use of bisphosphonates or other bone anti-resorptive drugs for modulating subchondral bone remodeling to prevent/slow the progression of osteoarthritic disease. Effects of ZA on articular cartilage are presumably expected to be a secondary process mediated through the alteration of subchondral bone metabolism and structure during the onset and progression of osteoarthritis (Dougados, Ayral et al. 1994, Manicourt, Altman et al. 1999, El Hajjaji, Williams et al. 2004, Hayami, Pickarski et al. 2004, Pelletier, Troncy et al. 2011). However, in the present study, it has been shown that the chondro-protective effects of ZA still presents without the presence of subchondral bone. Mechanical properties of the cartilage explants were significantly improved with the treatment of ZA. Therefore ZA can have direct beneficial effects on chondrocytes, which is the central hypothesis tested in this study.

To check the beneficial mechanisms of the bisphosphonates at the cellular or molecular level, $[Ca^{2+}]_i$ signaling was utilized as an indicator for cellular activities. In the mechanotransduction activities of chondrocytes, $[Ca^{2+}]_i$ signaling is often regarded as one of the earliest events under physical stimuli and the upstream of many anabolic and catabolic pathways (Guilak, Butler et al. 2014). Our results showed that bovine chondrocytes residing in their natural solid matrix can release spontaneous $[Ca^{2+}]_i$ signaling without physical stimuli. Although the mechanism of spontaneous calcium signaling in chondrocytes is not fully understood or confirmed, a few studies proposed several potential candidate pathways. The underlying oscillation in membrane potential driven by K^+ channels can activate the action potential and the influx of Ca^{2+} which initiates the spontaneous calcium signaling (Charles and Hales 1995). Calcium concentration in extracellular space is usually in the range of 10^{-3} M in cartilage (Parvizi, Parpura et al. 2002), while cytosol calcium concentration can be as low as 10^{-7} M (Matta and Zakany 2012). The 10,000 fold gradient of Ca^{2+} could also result in the calcium influx into cytosol to evoke calcium responses (Machaca 2011). In cartilage solid matrix, fixed negative charges on GAG chains results in an unique ionic environment for chondrocytes, which may also influence the resting membrane potential of chondrocytes and contribute to the spontaneous calcium signaling (Matta and Zakany 2012). Proper Ca^{2+} homeostasis, represented by the spontaneous $[Ca^{2+}]_i$ signaling in chondrocytes, might reflect the normal metabolic activities of *in situ* chondrocytes. Moreover, spatiotemporal features of $[Ca^{2+}]_i$ signaling is a direct indicator of the chondrocytes capability to maintain the $[Ca^{2+}]_i$ level under a dynamic environment with oscillating extracellular ion concentration, electric potential, mechanical loading, osmotic and oxidative stress. Statistical analysis from our previous study showed that the $[Ca^{2+}]_i$ responsive rate of chondrocytes has a positive correlation to the mechanical properties of cartilage explants after long-term culture (Zhou, Park et al. 2015). Although such statistical correlation cannot prove the immediate dependency of these two parameters, it is consistent with the findings in literature, *i.e.*, interrupted calcium signaling can affect the mechanical integrity of cartilage. It could be postulated that more calcium activities for the chondrocyte indicates healthier status. In this study, exposure to ZA supplemented medium significantly promoted the spontaneous calcium signaling in chondrocytes, especially during long term *in vitro* culture, which could prove the ZA's direct effects on chondrocyte. Interestingly, it was found that BPs can reduce the apoptosis of osteoblasts and osteocytes induced by glucocorticoids *in vitro* and *in vivo* without affecting osteoclast (Plotkin, Bivi et al. 2011). This protective effect is shown to be related to the connexin 43 hemichannels expressed on bone cells. Connexin 43 (Cx43) hemichannels on chondrocytes can be opened and release ATP by cyclic loading (Garcia and Knight 2010), which leads to activation of the kinases Src and extracellular signal-regulated kinases (ERKs). BPs could also have effects on this Cx43 hemichannels. The extracellular ATP is a major player in the intracellular calcium signaling, therefore the spatiotemporal characteristics of $[Ca^{2+}]_i$ intensity oscillations can be correlated with their metabolism status. ZA increased the frequencies of calcium activities, which could be postulated that ZA could facilitate the opening of Cx43 hemichannels under mechanical stimulation, leading to a cascade of downstream anabolic activities. Another study also revealed that chondrocytes could utilize the purinergic receptor pathway as part of the mechanotransduction cascade in which ATP is released through connexin 43 (Rosa, Collavino et al. 2014). A future research direction can be focused on ZA's effects on Cx43 hemichannels.

Anti-resorptive effect by nitrogen contained BPs (like Zoledronic acid) mainly results from the inhibition of the osteoclast mevalonate pathway. As for chondrocytes, our recent experiment showed the chondro-protective effect of BPs could also be related to the mevalonate pathway (results not shown). Our results demonstrated that supplement of mevalonate derivatives reversed the chondro-protective effects of ZA in terms of mechanical properties, which suggests that ZA may regulate the metabolic activities through the mevalonate pathway in chondrocytes, in specific, protein geranylgeranylation. Geranylgeranylation is required for the release of MMP-13, a major proteinase responsible for cartilage degeneration. This geranylgeranylation-produced degradation enzyme could also boost the $[Ca^{2+}]_i$ activities of chondrocyte, so that short-term treatment of geranyl-geraniol (GGOH) did not dampen the $[Ca^{2+}]_i$ activities. In long-term culture when the ECM is degenerated by the enzymes, mevalonate derivatives reversed the chondro-protective action of ZA in terms of mechanical integrity. The chondro-protective function by inhibiting the mevalonate pathway could be correlated with the reduced formation of Rac, Rho, Cdc42. Another possible mechanism may lie in the ATP-analog AppCCl2p, which is the metabolite in the middle of the mevalonate pathway. It was found that because of the structural similarities, AppCCl2p could act on the ATP purinergic receptor leading to an anabolic response (Rosa, Collavino et al. 2014). This could be the similar mechanism by direct stimulation of the purinergic receptors (P2Y) by ATP; which have been shown to elicit an anabolic response in both bovine and human chondrocytes (Croucher, Crawford et al. 2000).

4.5 Conclusions

Our *in vitro* cartilage explant model without the presence of subchondral bone showed that, for the first time, the bisphosphonate can exert direct chondro-protective action on chondrocytes. Supplement of ZA in culture medium can promote the beneficial effects of mechanical stimulation during cartilage explant culture. The $[Ca^{2+}]_i$ signaling of *in situ* chondrocytes is also significantly promoted by ZA, proving the direct effect of ZA on the metabolism of cells. The identified mechanisms is summarized in Figure 4.8.

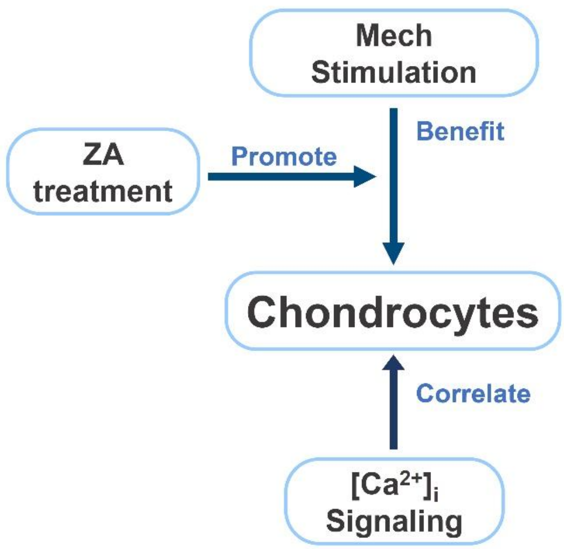

Figure 4.8 Summary of the ZA treatment with mechanical stimulation. ZA promoted beneficial effects of mechanical stimulation. Calcium signaling confirms the chondro-protective effect of chondrocytes and correlates with the previous results.

Chapter 5

EFFECT OF STATIN FOR THE PREVENTION OF PTOA

5.1 Introduction

Nitrogen-containing bisphosphonates (BPs) have been known for its anti-resorptive effect by the inhibition of the mevalonate pathway (Reszka and Rodan 2003, Reszka and Rodan 2004, Drake and Cremers 2010). Inhibition of enzymes in the mevalonate pathway prevents the prenylation process of proteins, such as Rho and ras, and leads to loss of cytoskeleton function which controls osteoclast morphology, formation, death, and survival (Coxon and Rogers 2003). In chapter 4, we have found that BPs could rescue the traumatic damaged chondrocytes, and the chondro-protective effect of BPs is also related to the inhibition of the mevalonate pathway. As an FDA approved medicine, BP could be a good candidate for prevention of PTOA. However, BPs also have serious adverse effects on the regular bone remodeling by messing up the bone resorption. Moreover, the gastroesophageal irritation, osteonecrosis of the jaw and severe suppression of bone turnover are all potential problems for PTOA patients with healthy bone (Kennel and Drake 2009). For young adults with functional growth plate, BPs can mess up the bone modeling process and cause severe abnormality in long bone. Therefore, it could be challenging to adopt BP for the prevention of PTOA patients, especially those who are young with healthy bone tissue. It is intuitive to look for an alternative drug that can have similar effect of BPs or target the same pathway on chondrocyte, while the strong side effect of BPs could be avoided.

Fortunately, BPs are not the only FDA-approved medicine targeting the mevalonate pathway in cells. Statin is a class of drugs that is widely used to lower cholesterol level and to prevent cardiovascular disease, which also target at the production of mevalonate (Pedersen, Kjekshus et al. 1996, Buhaescu and Izzedine 2007), as shown in Figure 5.1. Statin can accumulate in the liver, inhibit the mevalonate pathway in cells, and prevent the manufacturing of cholesterol. Stains are widely used in the U.S. for therapeutic purposes, and in 2003, atorvastatin became the best-selling pharmaceutical in history (Simons 2003). Statins are often prescribed to patients to reduce their cholesterol level. It is estimated that one in four Americans, ages 45 and older, are taking a statin (Peter Wehrwein 2011). The side effects of statin, although not common, include muscle pain, digestive problems, increased blood sugar, type 2 diabetes and liver damage. Interestingly, many previous *in vivo* and *in vitro* studies have shown that statin have chondro-protective effects, such as decreasing MMP-3 (Lazzerini, Capecchi et al. 2004), MMP-13 (Simopoulou, Malizos et al. 2010), IL-6, IL-8 and NO production of chondrocytes (Dombrecht, Van Offel et al. 2007). A prospective population-based cohort study, known as “Rotterdam study,” showed that statin use is associated with more than a 50% reduction in progression of the knee OA (cite “Statin use is associated with reduced incidence and progression of knee osteoarthritis…”). Similar beneficial effects of statin on arthritis were noticed in several other clinical trials, such as UK study and Israel study (cite “Statin Use and clinical Osteoarthritis in the general population: a longitudinal study.” “Use of statin is associated with a lower prevalence of generalized OA”, and “Persistence with statins and onset of rheumatoid arthritis: a population-based cohort study”). It is widely believed that the anti-inflammatory effect, which is due to the inhibition of NF-κB, of statin can benefit the joint health, especially for rheumatoid arthritis. However, few studies have connected the inhibition of mevalonate pathway with the joint beneficial effects of statins. Strong evidences from our previous studies in Chapter 4 proved that the chondro-protective function of BPs is related to its inhibition of mevalonate pathway in chondrocyte. Therefore it is natural to conjecture that statins may also have chondro-protective functions due to its inhibition of mevalonate pathway.

Therefore, in this study, we hypothesized that stain can directly regulate the metabolism of chondrocytes and rescue the cartilage from trauma damage. Testing of this hypothesis may provide a new pharmaceutical candidate for the prevention of PTOA. Compared to the BPs, statins have the following advantages when used for PTOA: 1) Statin has been widely used for a large population with mild and well-understood side effects. The new guideline from the American College of Cardiology and the American Heart Association will increase the number of U.S. adults (40-75 yrs old) receiving or eligible for statin therapy from 43.2 million (37.5%) to 56.0 million (48.6%) (Pencina, Navar-Boggan et al. 2014). 2) Unlike bisphosphonates, statins do not bind to bone and do not show strong effects on bone remodeling, making them safer to use on children (8 yrs old) (Stein 2007, Uzzan, Cohen et al. 2007) and young soldiers or veterans. 3) The cost of statins could be as low as $4 per month, and continuously decreases as the patents are expiring. 4) Statins have shown anti-inflammation effects by inhibiting the synthesis of NF-kB (Lazzerini, Lorenzini et al. 2007), and they can promote bone formation and counteract bone loss (Funk, Chen et al. 2008). Both joint inflammation and subchondral bone loss are potential risks for OA development after joint injuries. Indeed, a large body of evidence suggests that statins may favorably affect the joints, especially for those with rheumatic arthritis, by exerting a protective activity on synovium, cartilage, and subchondral bone (Hall 2003, Lazzerini, Capecchi et al. 2011).

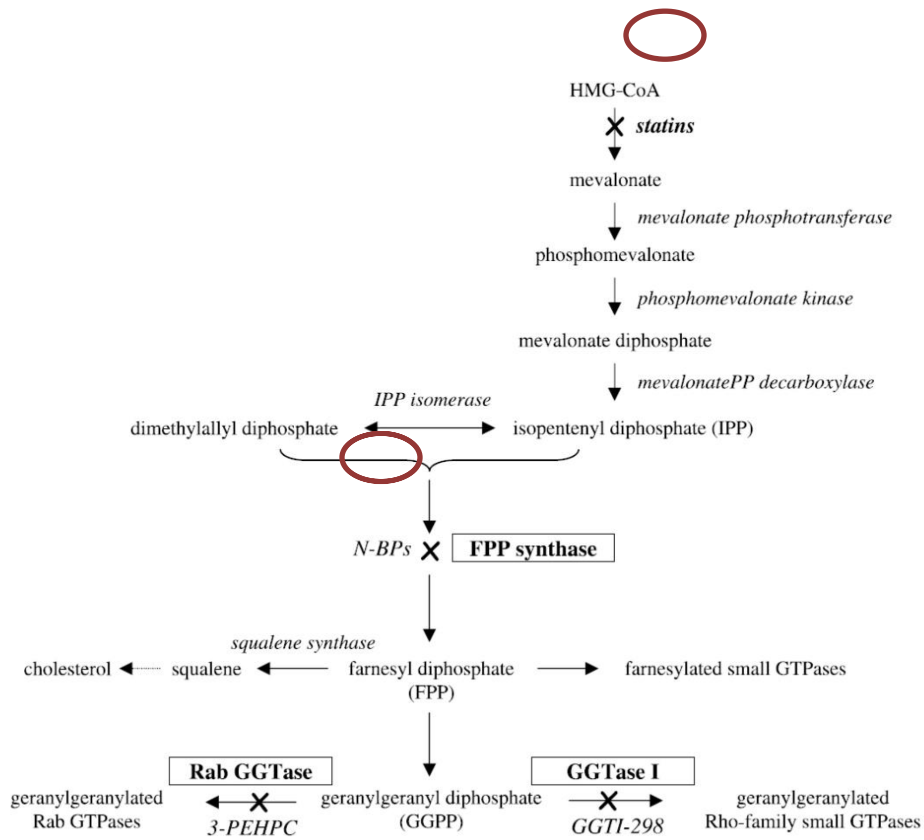

Figure 5.1 Schematic representation of the Mevalonate pathway and its inhibitors (Roelofs, Thompson et al. 2006).

This chapter will investigate the chondro-protective effects of statins using *in vitro* cartilage explant model similar to the studies in Chapter 4 using BPs. First, the cartilage was damaged with serum exposure, and then statin was supplemented in the culture medium to test whether it can rescue the chondrocytes from damage. Second, cartilage sample was exposed to inflammatory cytokine, and then rescued by long-term statin culture. These two studies will test the effects of statins on long-term cartilage explant culture and its effects on chondrocytes.

5.2 Methods

5.2.1 Cartilage Explant Harvest and Culture

Cylindrical cartilage explants were harvested from the femoral condyle head of 3~6 month-old fresh calf knee joints (Green village, NJ) using a 3 mm biopsy punch. Superficial-to-middle zone of the cartilage (2 mm in thickness) was obtained with a custom designed cutting tool. Cartilage samples were then cultured in medium (mentioned later) at 37 °C, 100% humidity and controlled CO_2 level (5%). Culture medium was changed every other day. Chondrogenic medium (DMEM, 1% ITS+ Premix, 50µg/ml L-proline, 0.1 µM dexamethasone, 0.9 mM sodium pyruvate, 50 µg/ml ascorbate 2-phosphate) was used to culture the cartilage allografts.

5.2.2 Effects of Statin

The cartilage samples were first balanced in culture medium for 3 days, and were then cultured in chondrogenic medium with or without 1 µM simvastatin for 24 hours (Sigma). The $[Ca^{2+}]_i$ signaling of *in situ* chondrocytes were measured using technique decribed in previous chapters. Using another batch of cartilage explants, the gene expression of chondrocytes was examined. The samples were first cultured in IL-1α (1ng/ml) supplemented culture medium for four days. The damaged the explants were then transferred to regular culture medium supplemented with or without statin for 1 week. Gene expression levels of Type I, II collagens and aggrecan of chondrocytes were measured.

5.2.3 Statistical Analysis

Two-way ANOVA with Bonferroni post-hoc analysis was used to compare the mechanical properties between different groups over culture time. Unpaired student's t-test (2-tail) was utilized to determine the significant difference between groups for the other parameters. All data are presented as mean ± standard deviation. Significance level was chosen at $p < 0.05$.

5.3 Results

5.3.1 Calcium Signaling of the Cartilage Explants after 24 hour treatment with 1 µM Simvastatin

Cartilage explants were cultured in 1µM simvastatin for 24 hours, and the calcium signaling of chondrocytes was analyzed. Significantly more calcium signaling was observed in simvastatin treated samples (24.3% vs. 31.6% in control and simvastatin groups, respectively) (Figure 5.2). The peak relaxation time of statin treated cells was much shorter than the control group, why the magnitude of calcium peaks are lower in the treated group. Thus after statin treatment, the chondrocytes recover from a calcium release earlier and faster when compared with untreated cells.

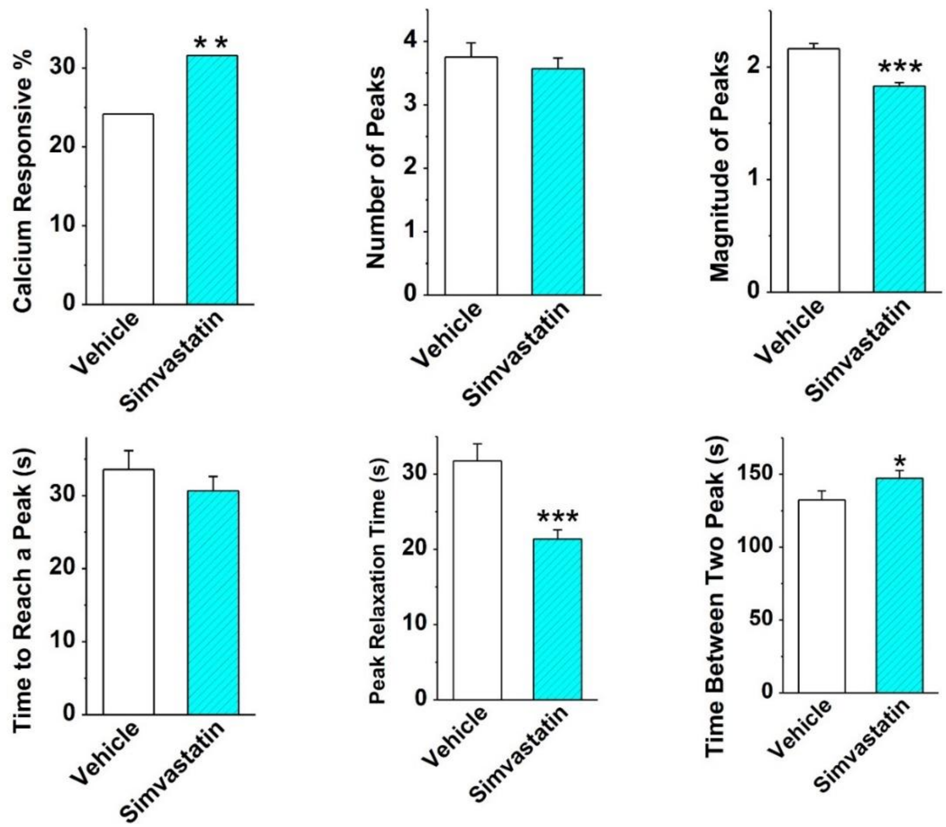

Figure 5.2 $[Ca^{2+}]_i$ responses of in situ chondrocyte after 24 hr 1µM simvastatin treatment. Calcium responsive percentage, number of calcium peaks, magnitude of peaks, time to reach a peak, 50% peak relaxation time and time between two peaks were analyzed (n>100 cells; *:p<0.05,**:p<0.01, ***:p<0.001, error bars: standard deviation).

5.3.2 Calcium Signaling of the Cartilage Explants after 24 hour treatment with 1 µM Simvastatin

Gene expression levels were checked on cartilage samples with 4 day IL-1α damage followed by 1 week simvastatin treatment, and significant up-regulation of type II Collagen (6.4 folds) and aggrecan (5.6 folds) were observed in statin treated group (Figure 5.3). No significant difference was detected for the expression of type I collagen.

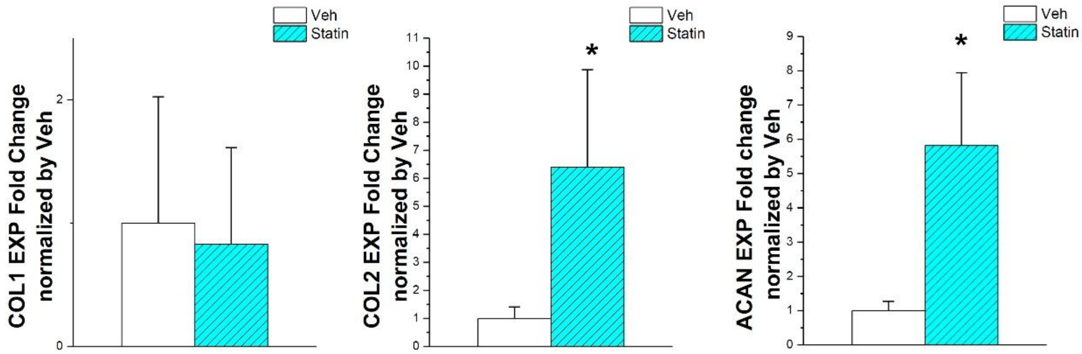

Figure 5.3 Gene expression of chondrocytes in cartilage treated with 1µM simvastatin after 1 week serum culture. GAPDH was used as a reference gene and genes of interests are Collagen type I, II and Aggrecan (*:p<0.05, n=6, error bars: standard deviation).

5.4 Discussion

Statin, the key rate-limiting enzyme of the mevalonate pathway, inhibits the enzyme HMG-CoA reductase in cells. This inhibition effect of HMG-CoA reductase has been employed to lower the cholesterol levels, prevent vascular and coronary heart disease, and reduce the amount of inflammation (Bellosta, Ferri et al. 2000, Waldman and Kritharides 2003, Calabro and Yeh 2005). These effects are also dependent on the inhibiting post-translational modification (isoprenylation) of the GTP-binding proteins of Ras superfamily (Laufs and Liao 2003). Statins have displayed multiple joint-protective effects, which are summarized in Table 5.1. Mevastatin increases the mRNA levels of BMP-2, aggrecan and type II collagen as well as increasing proteoglycan synthesis in rat chondrocytes (Hatano, Maruo et al. 2003). Yudoh K et al. has shown that statin has the potential to treat inflammatory arthritis (Yudoh and Karasawa 2010). It can function as the protective agents against chondrocyte aging and degeneration of articular cartilage during the progression of OA. The possible mechanisms of statin on cartilage could be dependent on reducing the production of matrix metalloproteinases (MMPs) (Luan, Chase et al. 2003, Thunyakitpisal and Chaisuparat 2004, Wilson, Evans et al. 2005).

Table 5.1 Effect of Statins on cartilage: *In vitro*, animal, and clinical studies

Cell Type	Conc.	Incubation time	Outcome	Authors
Synoviocytes	0.1 uM	24hrs	*in vitro* anti-inflammatory activity in RA	(Blaschke, Viereck et al. 2009)
OA Chondrocytes	10, 50 uM	24 hrs	50uM better	(Simopoulou, Malizos et al. 2010)
OA Chondrocytes	5, 10, 50 uM	48 hrs	dose-dependent	(Lazzerini, Capecchi et al. 2004)
Synoviocytes	0.05, 10 uM	12 hrs	high conc. Induce apoptosis; 50 uM 48hrs	(Yokota, Miyazaki et al. 2006)
Human Chondrocytes	0.5, 5, 10, 50 uM	48hr	dose-dependent	(Dombrecht, Van Offel et al. 2007)
OA firboblast-like synoviocytes	1, 5, 15 and 50 uM	48 hrs	higher conc more apoptosis	(Litinsky, Golan et al. 2009)
disc chondrocytes	0.3, 3 uM	7dy	Up regulation of ACAN, col2, bmp-2	(Zhang and Lin 2008)
Human Chondrocytes	1, 5 and 10 uM	18hr	Not significant between groups	(Baker, Walsh et al. 2012)

In chapter 4, we have shown that BPs have positive effects on chondrocytes in terms of biomechanical properties, biochemical composition and gene expression. Many studies revealed the joint beneficial effects of statin using *in vivo* studies, although most studies believed that it is related to the anti-inflammatory function of statins. According to the flow chart of mevalonate pathway shown in Figure 5.1, statin and BPs are both known of the inhibition of mevalonate pathway, while statin blocks the pathway more upstream than BPs. In this study, we found that statins have a positive effect on chondrocytes during *in vitro* culture. Short term treatment (24 hours) of statin showed promotion of the anabolic gene expression and cell calcium signaling. This finding is consistent with those in literature (Hatano, Maruo et al. 2003). Blocking the HMG-CoA reductase with statin could interrupt the protein prenylation (Lazzerini, Lorenzini et al. 2007). It is likely that statin inhibits the function of intracellular proteins that regulates the cytokine production (Corsini, Bellosta et al. 1999). The Rho family of GTPases has been recognized as the main geranylgeranylated protein that is associated with the activation of nuclear factor NF-κB (Perona, Montaner et al. 1997, Corsini, Bellosta et al. 1999). Previous studies have shown that the chondro-protective effect of statin may rely on its inhibition of Rho GTPases, the downstream of the mevalonate pathway. Members of the Rho family of small GTPases are considered as the key regulators of the actin cytoskeleton (Hall 1998). To verify the positive effect of statin on Rho GTPases, future study can check its effect on chondrocyte cytoskeleton, such as the mechanical stiffness of cells or the regulatory volume change of cells under osmotic stress (Bush and Hall 2001, Erickson, Northrup et al. 2003).

5.5 Conclusions

In summary, statin treatment showed beneficial effects on cartilage samples, including the promotion of spontaneous calcium signaling in chondrocytes and the upregulation of type II collagen and aggrecan gene expression levels. It remains elusive why the statin shows positive effect on cartilage *in vitro*, and how it regulates the upstream and downstream of mevalonate pathway in chondrocytes. Further studies are necessary to understand current results and the related molecular mechanisms.

Chapter 6

SUMMARY AND FUTURE DIRECTIONS

6.1 Summary

Post-traumatic osteoarthritis (PTOA) is a long-term adverse consequence of joint trauma such as meniscal or ligament tears. Cartilage degeneration starts after traumatic damage with unknown reasons. It has been conjectured that PTOA results from a combination of biochemical and mechanical insults on the cartilage. Although the OA development is often expected after joint trauma, there are a few preventive treatments available. Both non-pharmacological methods (e.g., diet control, change of shoe wedges, and physical therapy) and pharmacological methods (e.g., anti-inflammatory drugs and joint lubricants) have been widely adopted to treat the joints after traumatic injuries. However, these preventive treatments only provide some marginal improvement of joint functions. None has been shown to effectively prevent the PTOA initiation. Once the OA starts, there is no efficient medicine or therapeutic technique available to stop the progression of OA or reverse the cartilage degeneration. One of the aims of this thesis is to search for a new drug for the prevention of PTOA, and promising results have been revealed.

Since there is no neuronal or vascular system in cartilage, the diagnosis of cartilage degeneration at early stage remains challenging. Once the degeneration starts, cartilage has minimal ability to heal spontaneously, and the repair process is typically incompetent. The treatment of PTOA is very similar to those for primary OA. The earliest surgical intervention opportunity is to repair the small lesions on cartilage with microfracture surgery. Many microfracture studies yielded variable and unpredictable healing results, and there is a great need to improve the surgical outcome. However, due to the high cost and ethical concerns of large animal study and clinical trial, the tissue repair mechanisms of microfracture is not fully understood. Although some improvement techniques have been proposed for microfracture procedure, such as the use of porous scaffold and delivery of growth factors, little scientific evidence is available to support their application in clinical practice. The second aim of this thesis is to build an *in vitro* microfracture model, which can simulate the microfracture surgery on cartilage-bone explants, replicate the rehabilitation procedure and longitudinally measure biomechanical and biochemical properties of the repaired tissue at the lesion site. This new *in vitro* model could further enable the study of microfracture to benefit from the tremendous knowledge obtained in tissue engineering and stem cell research.

In chapter 2, a novel *in vitro* microfracture model was developed using long-term culture of 3D cartilage-bone explant. It has the advantage of simplifying the *in vivo* system while preserving all involved cells such as stem cells in bone marrow, osteoblast in bone and chondrocytes in cartilage. The most important feature of microfracture was replicated using this *in vitro* model, the formation of cartilaginous tissue by creating a bone marrow at the lesion site. Using this *in vitro* model, we demonstrated that supplement of TGF-β3 can significantly improve the quality of newly generated tissue for bone marrow. Thus chondrogenesis of the bone marrow derived progenitor cells are responsible for the new tissue generation after microfracture surgery.

Rehabilitation after the microfracture is known to be essential for the surgical outcome. There is a great need for well-conducted, statistically valid studies to address the void in relevant literature. To search for an optimized rehabilitation protocol, it may require the comparison of numerous rehab profiles with the combination of different starting time, duration, loading profile and loading magnitude. It is difficult to guide the large animal to follow designed rehab protocols. Thus the *in vitro* model built in Chapter 2 could be a perfect system for the study of microfracture rehabilitation. In chapter 3, using the *in vitro* microfracture model, we studied the effects of mechanical loading and different starting time of mechanical loading. As hypothesized, the mechanical loading significantly benefits the formation of new cartilage, especially at the middle-deep zone. Interestingly, our study showed that the system is sensitive to different mechanical stimulation profiles, and performed the delayed mechanical loading after microfracture procedure can promote the chondrogenesis of bone marrow cells, and improve mechanical integrity. This new finding provides important guidance for the design of rehab protocol, as more and more surgeons prefer immediate partial weight bearing on the knee joint after surgery.

Although various surgical strategies have been developed to repair cartilage lesion and to restore joint function at the late stage of PTOA, few techniques can prevent or postpone the initiation of OA after joint injuries. In chapter 4, the effect of bisphosphonates on the prevention of PTOA was investigated. A bold hypothesis was tested in this study. BPs can rescue PTOA by directly regulating the metabolic activities of chondrocytes. Using an *in vitro* culture model of cartilage explant, we found that supplement of ZA can promote the beneficial activities of mechanical stimulation. The drug can promote the expression of anabolic genes such as type II collagen and aggrecan, whilst suppress the expression of catabolic genes including ADAMTS5 and MMP13. Furthermore, it was found that ZA treatment modified the spontaneous calcium signaling of in situ chondrocytes in the cartilage explant. Since calcium is the universal second messenger for chondrocytes, our finding implies that ZA can regulate the fundamental activities of chondrocytes in cartilage explant. This study opened a new direction in the search of preventive techniques of PTOA.

The last study of this thesis, presented in Chapter 5, investigated another potential drug for the prevention of PTOA initiation. The major function of bisphosphonates is to inhibit the mevalonate pathway in cells, which is the upstream of the prenylation of proteins. As another FDA approved drug, stain is also targeting at mevalonate pathway, which is a widely used drug to reduce cholesterol for patients with cardiovascular disease. Interestingly, a number of studies showed that stain can also benefit the joint health by acting on several cell populations. Our study found that statin treatment has positive effect on chondrocytes. Short-term statin treatment can promote calcium signaling in the cells and upregulate the expression of type II collagen and aggrecan genes.

6.2 Limitations and Future Directions

6.2.1 *In vitro* microfracture model

After microfracture surgery, bone marrow and blood were pushed out of the subchondral bone and form a clot at the lesion site. In most cases, the clot cannot fill the lesion. Due to the lack of blood pressure, no clot can form automatically at the lesion site. Bone marrow from porous bone was harvested and filled manually at the lesion. Therefore the quality and amount of bone marrow used *in vitro* could be different than the actual *in vivo* situation. This limitation may have some effects on the outcome of the system. However, we do believe that the major conclusion should be valid.

With the rapid development of tissue engineering technology, a combination of microfracture and biomaterials, including both synthetic and natural polymers, have been introduced for cartilage repair (Hoemann, Hurtig et al. 2005, Erggelet, Endres et al. 2009, Christensen, Foldager et al. 2012). The scaffold provides a stable 3D structure that can sustain marrow clot, protect the BMSCs from over loading, and improve the niche for progenitor cells. Hydrogel is one of the most promising options for scaffold which has numerous desirable traits including high tissue-like water content, moldable characteristics, and biocompatibility (Elisseeff, Lee et al. 2002) (Wang, Stewart et al. 1999, Drury and Mooney 2003). They are insoluble, hydrophilic polymer networks formed by crosslinking monomers through covalent or hydrogen bonds, or van der Waals interactions between the monomer chains (Peppas 1987). The presence of water and the porous structure allows the influx of solutes and nutrients to the cells, as well as the transport of cellular waste out of the hydrogel (Torres, Freyman et al. 2000, Elisseeff, Lee et al. 2002). Moreover, the gel could be an excellent carrier of growth factors and release them in a controlled manner. Therefore, a future direction for the microfracture study is to test the beneficial effects of adding new hydrogel scaffold at the lesion site together with bone marrow. The convenience of *in vitro* system can allow the testing of various biomaterials, such as a combination of porous 3D material and control release particles loaded with TGF-β3. The results could provide important guidance for the improvement of microfracture procedure. Another important future direction is the study of microfracture techniques, such as the effect of lesion size, shape of lesion edge, size of fractures on bone. All these factors can be investigated using the *in vitro* system.

Partial weight bearing is often adopted immediately after microfracture surgery. The benefits of PWB is not only the mechanical stimulation on progenitor cells in bone marrow, but also the health of the other connective tissues in the joint. Therefore, a delayed mechanical loading may be eventually abandoned in clinical practice. Another future direction of the microfracture is to investigate the effects of different loading profiles during rehab, such as compression versus shear loading. It is also critical to identify the optimal loading magnitude on the marrow clot at different time points, as overloading can crash the neocartilage at the early stage, while insufficient loading may not be able to promote the nutrient transport into the middle-deep zone. All these factors can be studied using the new *in vitro* system and generate preliminary data for the design of future *in vivo* studies.

6.2.2 Prevention of PTOA

Inhibition of mevalonate pathway is the major mechanism of BPs effect on osteoclast. Although our study has proved that ZA can directly affect the metabolism of chondrocytes, it is not clear how the drug works on the cells. A natural future direction of this study is to investigate whether the chondro-protective function of ZA is related to the inhibition of mevalonate pathway. As mevalonate is required for many important cell activities, it is also essential to identify the downstream pathway responsible for its chondro-protective function, such as the generation of Rho, ras or the inhibition of cholesterol secretion. As the BPs have strong effects on the osteoclasts and bone remodeling, it could be challenging to use systematical administration of BP for the prevention of PTOA. A potential technique to avoid this disadvantage is the local delivery of BP into the injured joints. Some polymer could be used as carrier of the BP and be injected into the joint for long-term BP release.

Our study has shown that statin may have a biphasic effect on the chondrocyte during long-term *in vitro* culture. It is critical to verify this result using animal models. A number of *in vivo* studies and clinical trials showed that statin can benefit the rheumatoid arthritis. It is promising that statin may also benefit the PTOA patients, since it targets at the same pathway with BPs.

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