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CARTILAGE DEFECT REPAIR
BY OSTEOCHONDRAL ALLOGRAFTS:
ROLE OF TISSUE SURFACES AND INTERFACES

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Andrea L. Pallante

Committee in charge:

　　Professor Robert L. Sah, Chair
　　Professor William D. Bugbee, Co-Chair
　　Professor Christine B. Chung
　　Professor Andrew D. McCulloch
　　Professor Shyni Varghese

2012

The dissertation of Andrea L. Pallante is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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_____
Co-Chair

_____
Chair

University of California, San Diego

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VITA

2005	B.S., Biomedical Engineering University of Rochester, Rochester, New York
2006-2012	Graduate Student Researcher Cartilage Tissue Engineering Laboratory University of California, San Diego, La Jolla, California
2009	M.S., Bioengineering University of California, San Diego, La Jolla, California
2012	Ph.D., Bioengineering University of California, San Diego, La Jolla, California

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Pallante AL, Chen AC, Ball ST, Amiel D, Masuda K, Sah RL, Bugbee WD. The in vivo performance of osteochondral allografts in the goat is diminished with extended storage and decreased cartilage cellularity. *Am J Sports Med* (in press) 2012.

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ABSTRACT OF THE DISSERTATION

CARTILAGE DEFECT REPAIR

BY OSTEOCHONDRAL ALLOGRAFTS:

ROLE OF TISSUE SURFACES AND INTERFACES

by

Andrea L. Pallante

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2012

Professor Robert L. Sah, Chair

Professor William D. Bugbee, Co-Chair

Articular cartilage exhibits limited intrinsic healing, and when defects are left untreated, they can increase in size and cartilage degeneration may progress to end-stage joint failure. Osteochondral allografting has been widely used in the clinic to treat large or multiple focal defects, or as a salvage procedure to avoid total joint replacement, and aims to restore mature, hyaline cartilage in a biologically, structurally, and functionally appropriate manner. Repair outcomes may be influenced by the presence of viable chondrocytes within graft cartilage and the integration of the graft bone to surrounding host tissue. Thus, the overall motivation of this dissertation

work was to develop an integrative and multi-scale approach to cartilage defect repair analysis by osteochondral allografts in order to expand the current understanding of *in vivo* cartilage and bone remodeling.

A novel approach for analyzing *in vivo* cartilage defect repair by osteochondral allografts was developed using interdisciplinary and multi-scale analysis methods in the goat model. Maintenance of cartilage load-bearing function in allografts *in vivo* was associated with zonal maintenance of cartilage cellularity and matrix content. Reduced cellularity at the articular surface, resulting from 4°C storage, was associated with variable long-term outcomes, while allograft failure was accompanied with cartilage softening, loss of cells/matrix, and/or graft subsidence. Production of the lubricant, proteoglycan-4 (PRG4), from allografts was a useful marker of biological performance. 37°C storage supports long-term chondrocyte viability, especially at the vulnerable articular surface. Bone structure in allografts was altered *in vivo* compared to non-operated bone, with allografts displaying bone cysts, and bone surface channels with or without roughening at the bone-cartilage interface.

This work has further elucidated the interrelationship between biological and structural aspects of cartilage and bone remodeling *in vivo* after osteochondral allografting. Such analysis has established the inferiority of 4°C stored allografts versus fresh allografts, identified potential biomarkers of allograft performance and alternative storage protocols, and provided insight into the pathogenesis of subchondral bone cysts during cartilage defect repair. Analyzing cartilage and bone biology, structure, and function in an integrative manner identified properties of the osteochondral tissue that are critical to repair efficacy and potential mechanisms of graft success/failure.

CHAPTER 1:

INTRODUCTION

1.1   General Introduction to the Dissertation

Articular cartilage exhibits limited intrinsic healing, and when defects are left untreated, they can increase in size and cartilage degeneration may progress to end-stage joint failure. Surgical interventions range from palliative to restorative, with variable repair outcomes. Fresh osteochondral allografting has been widely used in the clinic, with relatively long and successful history, to treat large or multiple focal defects, or as a salvage procedure to avoid total joint replacement. However, with the advent of additional screening and processing protocols delaying distribution of osteochondral allografts to surgeons, osteochondral donor tissue is now commonly stored at 4°C for 14-28 days. Chondrocyte viability deteriorates with increasing 4°C storage duration, but the long-term *in vivo* performance of such 4°C stored allografts is unknown. In addition, clinical repair outcomes are influenced by graft-host bone integration, and failure is often due to inadequate osseous incorporation leading to revision or conversion to total knee arthroplasty. The future application of osteochondral allografts may benefit from examining the relationship between cartilage integrity and subchondral bone remodeling during repair.

The overall motivation of this dissertation work was to develop an integrative and multi-scale approach to cartilage defect repair analysis by osteochondral allografts in order to expand the current understanding of *in vivo* cartilage and bone remodeling.

In vitro and *in vivo* experimental studies were conducted in the goat, a useful large animal model for studying osteochondral defect repair because the goat exhibits limited intrinsic healing. The objectives of the dissertation were 1) to validate the large animal model of osteochondral allografting and to describe the progression of cartilage success/failure after repair with fresh and frozen osteochondral allografts, 2) to determine the role of decreased cartilage cellularity at the surface on long-term *in vivo* cartilage repair outcomes, and 3) to elucidate mechanobiological mechanisms of cartilage and bone remodeling in order to improve overall repair efficacy (Fig. 1.1).

Chapter 1 of the dissertation begins with a review of clinical strategies to repair cartilage defects, osteochondral allograft composition and structure, and clinical osteochondral allograft efficacy. The chapter continues by describing potential animal models to study cartilage repair, which may be useful to examine mechanisms of allograft success or failure. Finally, the chapter ends with a discussion on mechanobiology of the cartilage surface and integration of interfaces within osteochondral allografts.

Chapter 2, which is in press in *Journal of Bone and Joint Surgery* [90], describes the effect of fresh versus frozen osteochondral allografts on the properties of articular cartilage after 6 months *in vivo* in the goat. A multi-disciplinary approach to cartilage defect repair analysis was applied to examine allograft performance, assessing osteochondral structure, zonal cartilage composition, and cartilage biomechanical function. Maintenance of cartilage load-bearing function in fresh osteochondral allografts was associated with zonal maintenance of cartilage cellularity and matrix content. Frozen osteochondral allografts displayed signs of failure at 6 months with cartilage softening, loss of cells and matrix, and/or graft subsidence.

Chapter 3, which has been submitted to *Journal of Orthopaedic Research* [89], describes the effect of frozen versus fresh osteochondral allografts on the secretion of functional proteoglycan-4 (PRG4) after storage and after 6 months *in vivo* in the goat. PRG4 secretion was evaluated as a potential biomarker of allograft performance and mechanism that protects the articular surface of allografts during repair. The results demonstrate that the osteochondral allograft storage conditions that affect chondrocyte viability (i.e. frozen) also affect the subsequent secretion of functional PRG4 secretion, not only immediately after storage, but also after implantation and retrieval 6 months post-operatively. PRG4 biosynthesis was a useful marker of allograft biological performance.

Chapter 4, which is in press in *American Journal of Sports Medicine* [88], describes the effect of 4°C storage on the properties of articular cartilage in osteochondral allografts after 12 months *in vivo* in the goat, and assesses the relationship between chondrocyte cellularity and allograft performance. Repair outcomes in 4°C-stored osteochondral allografts were inferior to fresh osteochondral allografts, and were accompanied by diminished cellularity at the surface, matrix fixed charge, and deterioration of cartilage structure. Cellularity at the articular surface was correlated with indices of cartilage health.

Chapter 5, which will be submitted in full to *Cartilage*, further explores the role of the cartilage surface in osteochondral allograft repair with 4°C stored allografts, building upon the results of Chapter 4, which identified superficial cellularity as a critically important factor related to allograft performance, and the methods of Chapter 3, identifying PRG4 as a potential biomarker of allograft performance. PRG4 secretion was diminished with increasing 4°C storage duration prior to implantation. Also, indices of *in vivo* repair efficacy, from Chapter 4,

correlated significantly with subsequent PRG4 secretion in retrieved osteochondral allografts after 12 months *in vivo* in the goat.

Chapter 6, which was published in *American Journal of Sports Medicine* [87], describes an alternative storage condition at physiological temperature (i.e. 37°C) for osteochondral allografts as a potential method to prevent storage-associated chondrocyte death. 37°C storage of osteochondral allografts supported long-term chondrocyte viability, especially at the vulnerable and superficial zone of cartilage.

Chapter 7, which will be submitted in full to *Journal of Bone and Mineral Research*, examines effect of cartilage defect repair by stored osteochondral allografts on bone structure after 12 months *in vivo* in the goat. Bone structure in OCA was altered compared to Non-Op joints with OCA displaying bone cysts and bone surface channels, and that bone structure was strongly related to cartilage properties and associated among subchondral, trabecular, and peri-cysts regions. The results of this bone and cyst structural analysis provide insight into potential mechanisms contributing to the development of bone cysts following cartilage defect repair by osteochondral allografts.

Finally, Chapter 8 summarizes the major findings and discusses future directions of this work.

1.2   Clinical Strategies to Repair Cartilage Defects

Articular cartilage has a limited capacity for self-repair; as a result of damage, by traumatic injury or progressive degenerative joint diseases, patients typically experience pain, decreased joint function, and often require surgical intervention to alleviate symptoms [13]. Cartilage defects are common and often symptomatic. During arthroscopies of symptomatic knees, 19% of patients had focal chondral or osteochondral defects, ranging in size from <1-4 cm^2, and typically located in load bearing regions of the joint [28, 49]. If such defects are left untreated, the lesions tend to increase in size [20, 56, 120] and may progress to osteoarthritis [110].

Surgical treatments articular cartilage defects aim to provide pain relief and improve joint function. Interventions range from palliative (arthroscopic debridement and lavage) to reparative (marrow stimulating techniques) and restorative (autologous chondrocyte implantation and osteochondral grafting) options (Fig. 1.2) [22]. Marrow stimulating techniques, including microfracture, are indicated for small (<2cm^2) chondral defects, and are often used as an initial treatment option due in part to its technical simplicity and short-term effectiveness [41]. Cell transplantation techniques, including autologous chondrocyte implantation (ACI) and matrix-assisted ACI (MACI), are indicated for uni-polar, well-contained medium-sized (2-10cm^2) chondral or osteochondral defects, with minimal bone loss (<6mm), and are often a second-line treatment [10]. Osteochondral autograft transplantation is indicated for small (<2cm^2) osteochondral lesions, and is limited by the amount of donor tissue available [47].

Osteochondral allografting is indicated for large chondral or osteochondral (>2cm^2) defects, and restores mature, hyaline cartilage structure at implantation. Young, active patients are suitable candidates [3, 21, 44, 64]. Additionally, osteochondral allografting is performed when other surgical techniques, such as

marrow stimulation and cell transplantation, have failed previously or are contraindicated [21, 64]. Osteochondral allografts have been used for treatment of focal cartilage defects [63, 73, 125], osteochondritis dissecans [31, 39], as well as post-traumatic, osteonecrotic, and bipolar lesions in the knee [7, 18, 74]. For transplantation of allografts, as with any allogenic organ or tissue, potential disease transmission and immunogenicity are important considerations [3, 24, 43, 44, 64]; however, the limited availability of suitable grafts, due in part to storage duration, restricts widespread application [43, 44].

1.3   Structure and Composition of Osteochondral Allografts

Osteochondral allografts repair large articular cartilage defects by restoring mature, hyaline cartilage in a biologically, structurally, and functionally appropriate manner. During such surgical procedure, a small arthrotomy is made to expose the cartilage defect (Fig. 1.3A), and the lesion site is prepared by removing diseased cartilage and a thin layer of bone. Then, an orthotopic osteochondral plug is harvest from a size-matched cadaveric donor to match the circumference and depth of the defect (Fig. 1.3B). The amount of allograft bone is typically limited to a few millimeters, which is sufficient to provide stable fixation, but minimizes the volume of transplanted bone. The donor allograft is carefully inserted (typically using short, gentle blows) by mallet impaction (Fig. 1.3C) until the cartilage surface is flush with the surrounding host cartilage (Fig. 1.3D) [14, 54].

The donor osteochondral allograft is a composite of full-thickness, mature, hyaline cartilage attached in its native form to its underlying bone. Currently fresh graft tissue is stored at 4°C to accommodate federal regulations that require tissue banks to screen and test donor tissue for infectious diseases before implantation [1]. For short 4°C storage durations, the cartilage is maintained in a biologically viable, but quiescent state, one in while chondrocyte metabolism is lower than normal [6, 9]. The non-living osseous component functions as an underlying support structure, similar to other types of bone grafts, fixing the graft to the host tissue, and acting as a scaffold for healing to the host by creeping substitution [43]. The composite graft forms an intact structural and functional unit to replace diseased osteochondral tissue.

The limited availability of suitable allografts, due in part to storage duration, restricts widespread application [43, 44]. Donor screening and processing protocols typically require 14 days [44, 63, 125]; thus, osteochondral allografts are used after

prolonged storage on average 24 days after harvest (range 15-43 days) [44, 63, 73, 125]. During prolonged storage at 4°C, chondrocyte viability deteriorates with a substantial decrease by 28 days and with a decrease to as low as 40% by 14 days [6, 27, 30, 92, 93, 117, 123, 124, 127]; such deterioration has led to recommended actions for a maximum duration of graft storage ("shelf-life") and an implantation time within 30 days of harvest [63, 73]. Alternative storage conditions that maintain chondrocyte viability for a longer duration may lead to an increase in "shelf-life" and in turn, the availability of osteochondral allografts to surgeons and their patients.

1.4   Clinical Efficacy of Osteochondral Allografts

Long-term allograft efficacy may be dependent on sufficient chondrocyte metabolism to preserve cartilage homeostasis and prevent degeneration. Traditionally, osteochondral allografts were implanted fresh, within 7days, have high chondrocyte viability, and retrieved grafts have contained viable chondrocytes up to 29 years after implantation [23, 57, 72, 76, 126]. Usage of such fresh allografts has resulted in long-term clinical success rates exceeding 75% [7, 14, 18, 31, 74]. In contrast, frozen allografts have been used in for massive oncologic reconstructions after limb-salvaging resection of joint tissue due to tumors [65, 69]. Such frozen grafts are devoid of viable chondrocytes, and specimens retrieved at 8 months to 5 years after reconstructive surgery generally display degenerate and acellular cartilage [33, 34].

Currently in the United States, osteochondral allografts are stored at 4°C to accommodate commercial screening and processing protocols, with testing typically requiring at least 14days[1, 63, 125], and such 4°C storage may lead to inferior repair outcomes in patients. Such fresh-stored osteochondral allografts are routinely used after 4°C storage for prolonged durations (15-43days), which compromises chondrocyte viability, and has resulted in some short-term (average 36 months, range 21-68 months) clinical improvement compared to pre-operative function for small cohorts of patients (n=10-25) [29, 63, 73, 125]. However, long-term *in vivo* performance of these fresh-stored allografts, in association with variable cellularity, is unknown.

Repair outcomes may also be affected by subchondral bone integration as determined by remodeling of both the allograft and host. Allograft procedures can fail as a result of nonunion, late fragmentation, and graft collapse due to insufficient osseous support [18, 44, 61, 83, 109, 126]. Fresh allografts retrievals often

demonstrated that graft failure was associated with limited osseous incorporation, graft collapse, osteonecrosis, and pannus formation [126]. Such osseous integration, influenced, in part, by the immunogenicity of allogenic bone, has been improved by reducing surface antigens (by freezing) [36, 102] or matching leukocyte-antigens, with accompanying improvement in clinical outcomes [37, 111]. However, detailed examination assessing the relationship between cartilage integrity and bone healing have not been investigated.

1.5   Animal Models to Evaluate *In Vivo* Repair Outcomes

Animal models allow for a systematic and temporal evaluation of cartilage repair and remodeling. Data obtained from human allograft retrievals is skewed toward poor outcomes, since analysis is performed on "failed" cases, where revision or conversion to total joint arthroplasty was required [61, 83, 126]. Clinical evaluations based on patient satisfaction and often limited by availability for long-term follow-up, are not sensitive to early stage degeneration. Intra-disciplinary and multi-scale analyses of cartilage repair in animal models may elucidate tissue changes indicative of early stage degeneration, and provide insight into the time-course of cartilage remodeling and/or deterioration.

While the ideal model system representative of the human condition is not attainable, several suitable large animal studies are useful for studying chondral and osteochondral defect repair. Carefully identifying the scientific goals of the study is critical for selecting the appropriate animal to use and interpreting the resulting repair outcomes. Several factors to consider when choosing a model system include animal age and cartilage maturity, joint size and cartilage thickness, feasibility of non-weight bearing and rehabilitation protocols, and the ability to monitor long-term progression of repair efficacy (Table 1.1) [19, 52].

Animal models commonly used in osteochondral allograft studies include lapine [4, 35, 71, 103], canine [42, 68, 84, 101, 113], ovine [53, 95, 104, 119], caprine [55, 108], and equine [91] models. The rabbit (lapine) model has high potential for spontaneous healing, relatively thin cartilage, and sizeable variation from human loading conditions, but is useful for screening potential treatments and proof of concept or safety studies [2, 19, 52, 96, 121]. The dog (canine) model is limited by the size of the defects and generally thin cartilage, but lacks intrinsic repair response

similar to humans and is well-suited for specific exercise and rehabilitation protocols [2, 19, 52]. Goats (caprine) and sheep (ovine) are feasible long animal models for studying chondral and osteochondral defect repair as they exhibit limited intrinsic healing, and with goats having thicker cartilage than sheep, but lesion size typically studied is small and the subchondral bone plate is thick compared to humans [2, 19, 52, 56]. The horse (equine) model has large joint dimensions, making it suitable for arthroscopic assessment, thick articular cartilage, and fully extended upright stifle joints during gait, which makes it advantageous for studying chondral and osteochondral defect repair. However, high joint loading conditions, inability to maintain protected weight bearing protocols, and requirement of highly specialized research centers and care pose potential limitations [19, 45, 52].

Animal studies, which more closely investigate the effect of storage duration on allograft performance, could provide a systematic approach to evaluating the biological and structural properties of retrieved allografts, and also elucidate the role of chondrocyte viability on allograft efficacy. Results in animal models of fresh and frozen osteochondral allograft repair suggest that post-operative time is an important determinant of the quality of cartilage repair. Frozen allografts appear grossly normal, and similar to fresh allografts at short post-operative durations up to 3months [35, 53, 71, 104], and then appear discolored and roughened, often exposing subchondral bone, at 11-12months [53, 95, 104, 113]. In contrast, cartilage of fresh allografts remains grossly normal up to 12months, with only mild degenerative changes [91, 95, 113]. Gross observations are paralleled by histopathologic changes, including reduced (for frozen) and maintained (for fresh) Safranin-O staining intensity [4, 35, 42, 53, 67, 71, 84, 91, 95, 101, 104, 113]. Cartilage cellularity and load-bearing function have been analyzed in relatively few studies. Qualitatively, frozen allografts appear acellular by

3months, compared to fresh allografts, which contain viable cells up to 12months [53, 91, 95, 104, 113]. Confined compressive stiffness was slightly reduced with fresh allografts compared to non-operated controls, and markedly reduced with frozen allografts with increasing post-operative duration [42, 53, 84, 95, 104]. However, large animal studies with frozen versus fresh allografts at moderate time points (between 3-11months) are needed to understand the progression of cartilage deterioration.

Determinants of long-term allograft function can also be pursued using multi-scale analyses of cartilage repair in animal models. In animal models of 4°C stored osteochondral allografts, allografts stored at 4°C for 14days appear grossly, biochemically, and biomechanically similar to fresh allografts at short post-operative durations up to 3months [84], but exhibit mild to moderate gross degenerative changes at 6 and 12months [95, 108]. Allografts stored at 4°C for >21days have variable outcomes; some grafts tend to undergo severe degeneration, while others tend to be similar in appearance to fresh grafts and grafts stored at 4°C for 14days [67, 95]. At 12months, some 4°C stored allografts appear qualitatively to have diminished cellularity at the cartilage surface [95], which may be an important predictor of long-term graft efficacy. The reduction of cartilage load-bearing function was associated with proteoglycan content [95]; While storage duration did not correlate with histologic appearance at 12months [95], other determinants of repair outcomes have not been investigated. Rapid indentation of cartilage, which is sensitive to structural measures of cartilage degeneration [5] may be useful to elucidate mechanisms of allograft deterioration or maintenance. Identifying determinants of cartilage surface function may indicate early-stage cartilage deterioration and lead to future improvement in the application of OCA. In addition, quantitative analysis of

cellularity in retrievals and its relationship to graft efficacy may expand the current understanding of *in vivo* maintenance of cartilage structure, composition, and function.

While cartilage repair outcomes are generally good following osteochondral allograft repair, the structure of the underlying bone support has some abnormalities in animal models, which is consistent with clinical outcomes. Subchondral bone cysts, well-demarcated and often rounded areas of translucency in bone, can develop following osteochondral allografting [8, 95, 119], and other cartilage repair treatments [16, 17, 40, 86]; however, the relationship between cartilage and bone repair outcomes in unclear.

1.6   Mechanobiology of the Cartilage Surface

Restoration of cartilage surface biology and function following cartilage repair would be advantageous for long-term biological performance, as early stage cartilage degeneration is typically evident at the articular surface. Articular cartilage exhibits zone-dependent composition, structures, and functions [51]. The superficial zone, or zone closest to the articular surface, contains a high density of chondrocytes with flattened morphology [114], relatively low glycosaminoglycan (GAG) content, and collagen fibers running parallel to the articular surface [51], while also interfacing with synovial fluid (SF) present within the joint, which functions as a biomechanical lubricant of the articular surface, as well as the nutrient supply for the adjacent cartilage. Cartilage surface health may be a critical indicator of long-term function, and examining zone-specific functions, especially in the vulnerable surface region, may provide additional insight into the factors necessary for graft success or failure.

The superficial zone mediates lubricant function and allows opposing surfaces to distribute load across the joint surface. During boundary-mode lubrication, the load is supported by surface-to-surface contact, and lubricant molecules present in SF and secreted by superficial zone chondrocytes adhere to the articular surface, facilitating smooth joint articulation [50, 129]. The relatively low GAG content of the superficial zone contributes to its low compressive stiffness [105] and allows opposing surfaces to distribute forces from loading over a broad area, which reduces contact stress.

Biosynthesis of the lubricant molecule proteoglycan-4 (PRG4), which is secreted by chondrocytes in the superficial zone [107] and also found in SF [115], adsorbs to the articular surface in synovial joints, and is important for maintaining the health of the cartilage and joint [59, 60]. PRG4 has friction-lowering boundary lubrication ability [106], and contributes to the maintenance of cartilage homeostasis

and the preservation of normal joint function. PRG4 biosynthesis exhibits mechanosensitivity [46, 79-82, 128], indicates cartilage surface health [59, 60, 100], and may be a useful biomarker in cartilage repair, as it depends on the presence of chondrocytes at the articular surface and represents normal surface biology.

1.7   Integration of Host and Graft Interfaces in Osteochondral Allografts

Complete integration between the implant and host cartilage typically does not occur following repair by osteochondral grafts, leaving a laceration or channel through the cartilage surface to the underlying subchondral bone. During joint loading, pressurization of interstitial fluid within cartilage shields the solid matrix of cartilage from excessive strain [112]. Maintenance of fluid pressurization within cartilage following allograft implantation may be related to the ability of the implant-host interface to create an effective seal at the articular surface. Finite element models of a perforation through the subchondral bone resulted in insufficient fluid pressurization of the cartilage in the neighborhood of the perforation, increased solid stress on the matrix, and drove interstitial fluid toward the perforation [78]. Excessive stress on the solid matrix at the interfacial regions may contribute to matrix damage, and further reduce the sealing of the cartilage surface. Additionally, SF transport through the laceration may inhibit integrative repair and contribute to abnormal pathology within the subchondral bone, as lubricant molecules in SF, such as PRG4, have been implicated as a potential inhibitor of integrative repair [32]. Cyst-like lesions also often develop at the junction between osteochondral grafts and host tissue [119], and may connect to the overlying cartilage from a channel due to incomplete repair. Preventing fluid flow from entering the interface region of the implant-host cartilage and/or through the bulk cartilage during loading due to its depressurization may be necessary for the proper repair of both the cartilage and subchondral bone.

Bone is a mechanosensitive tissue which alters its internal architecture to adapt to the loads it experiences. During osteochondral allografting, the osseous portion of the graft acts as a scaffold for the host bone to invade and remodel. Distribution of

loads to the underlying graft bone may be essential to provide the appropriate anabolic strain signals to recruit bone cells necessary for remodeling. Appositional bone formation occurs by depositing new bone on existing bone [12], and typically dominates the type of bone formation occurring during allograft bone integration. Osteoblasts align on the bone surface and synthesize osteoid, the unmineralized and organic portion of the bone matrix, prior to maturation. The resulting woven (fiber or primary) bone has irregular collagen fibril orientation and mineralization, resulting in a more flexible, easily deformable, and weaker bone than lamellar (secondary) bone. During successful remodeling, woven bone is resorbed and replaced by mature, lamellar bone [11]. Presence of woven [118] and fibrotic [71, 84, 113] bone following cartilage repair, in addition to the cystic lesions [8, 95, 119], may indicate incomplete integration of the graft bone to the host.

While identification of subchondral bone cysts are evident following cartilage repair, the etiology of such subchondral bone pathology is unclear, particular in the setting of osteochondral allografts. Two hypotheses for cyst formation are (1) degenerative changes of the overlying cartilage, leading to aberrant communications between the joint space and subchondral bone, forcing pressurized synovial fluid exudation into the subchondral bone, which counteracts the compression loading in bone and results in net bone resorption (Synovial Fluid Intrusion) [25, 62], and as (2) a biological reaction of the subchondral bone in response to excessive, concentrated loads on the bone, leading to decoupled bone metabolism favoring bone resorption over formation (Bony Contusion) [85]. Examining subchondral plate discontinuities, cartilage pressurization, and bone structure may help to elucidate mechanisms of subchondral bone remodeling, and its relationship to articular cartilage properties, following osteochondral allografting.

Structural evaluation of cysts and the surrounding bone microarchitecture, in addition to mapping communication channels and sites of cartilage depressurization would be useful to analyze cyst pathology and etiology following cartilage repair. Cysts are defined, on MRI as well-demarcated regions with signal intensity similar to joint cavity fluid [15], and on μ(CT)/histology as substantial regions devoid of trabecular bone [86]. Cyst size is often measured in the image slice with the greatest lesion size, semi-quantitatively [58, 66, 86, 94, 116], or quantitatively as the maximum diameter in a selected plane [75] and/or as volume estimated from three orthogonal slices [15]. Subchondral plate discontinuities, allowing direct communication between the joint space and trabecular bone have been identified [48, 62, 75, 98, 99]; however, the extent of such discontinuities and connection pathways has not been elucidated. Bone microarchitecture around cysts is dense at the walls and then more porous and branched, with increased porosity of the subchondral plate, with a normal overall bone volume fraction [17, 77]; methods for regional bone evaluation have not yet been applied to cysts [70]. Articular cartilage dysfunction (decreased volume, histopathological indices of degeneration, decreased stiffness) is common, especially in peri-articular regions [15, 26, 75, 97, 116]; however, the extent of cartilage depressurization and its relationship to the subchondral region is unclear.

Table 1.1: Comparison of animal models for cartilage defect repair [19, 38, 52].

	Lapine	Canine	Ovine	Caprine	Equine	Human
Age of Maturity [months]	8	24	24	24	24	Adult
Cartilage Thickness [mm]	0.3	0.6-1.3	0.4-1.2	0.7-2.0	1.5-2.0	2.2-2.5
Defect Diameter [mm]	3	4	8	6	10+	10+
Non-Weight Bearing Protocols	—	—	+	+	—	+
Rehabilitation Protocols	—	+	—	—	+	+
Arthroscopic Assessment	—	+	+	+	+	+
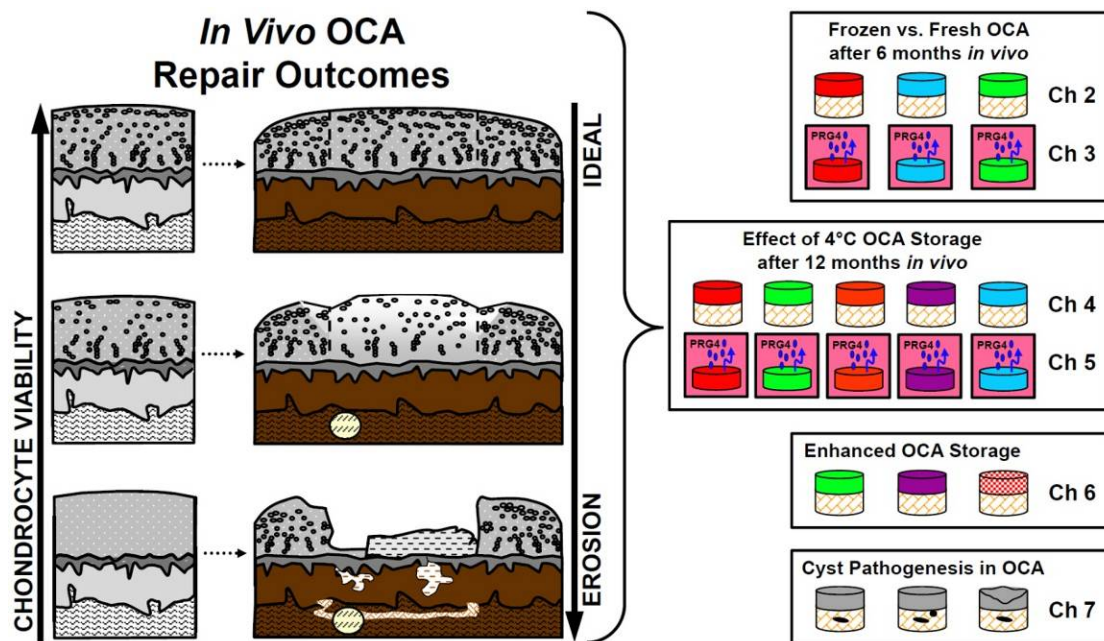
Figure 1.1: Overall aims of the dissertation.

small defects   **medium defects**   **large defects**   **end-stage degeneration**

Microfracture   **ACI / M-ACI**   **Mosaicplasty**   **allograft**   **TJR**
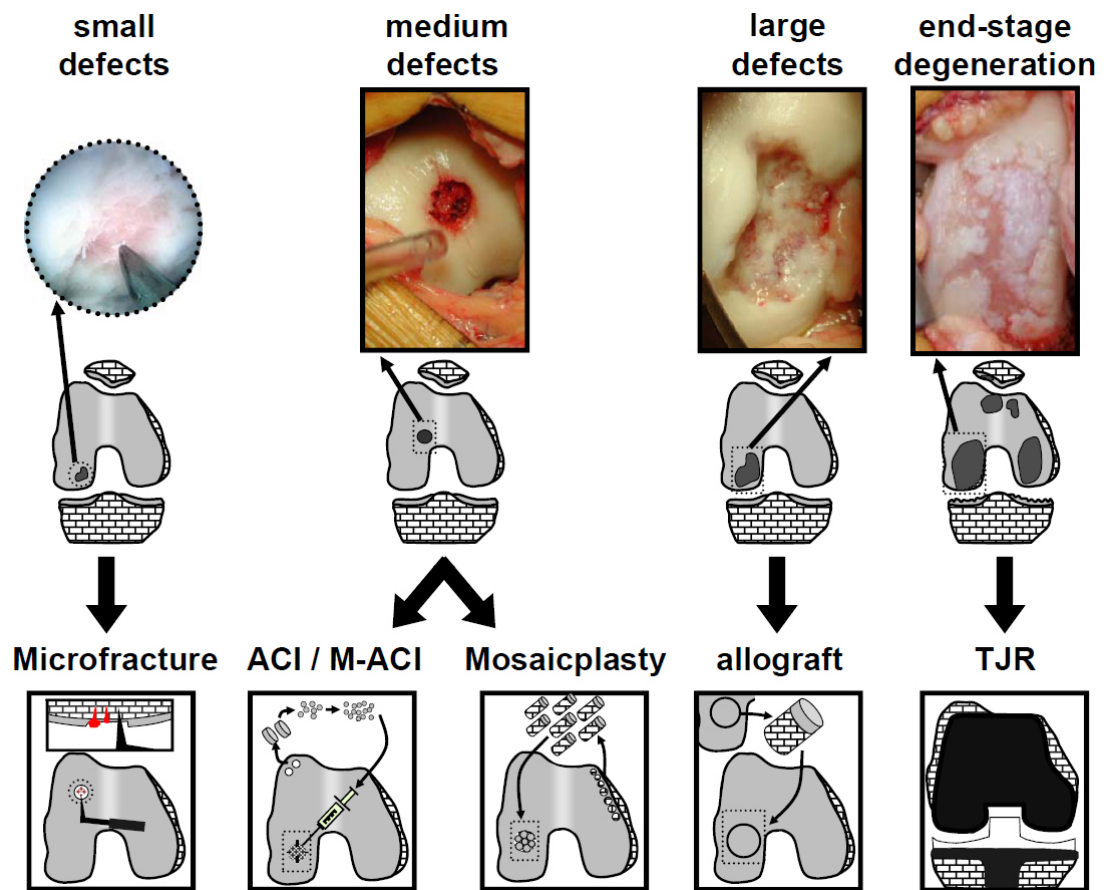
Figure 1.2: Cartilage defects and treatments. Surgical treatments vary with lesion size. Adapted from [122].
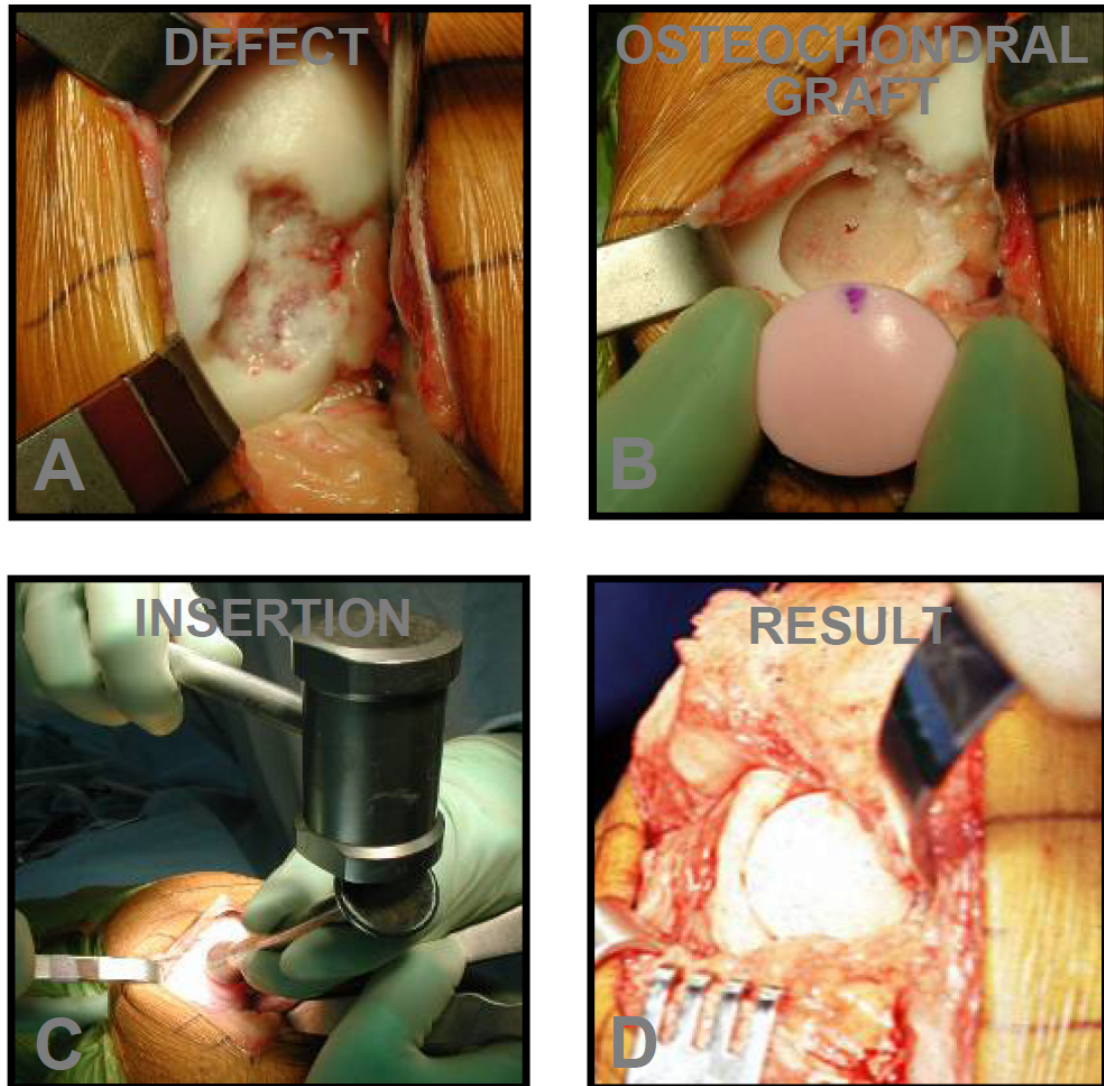
Figure 1.3: Surgical treatment of cartilage defects with osteochondral. Adapted from [54].

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CHAPTER 2:

TREATMENT OF ARTICULAR CARTILAGE DEFECTS IN THE GOAT WITH FROZEN VERSUS FRESH OSTEOCHONDRAL ALLOGRAFTS: EFFECTS ON CARTILAGE STIFFNESS, ZONAL COMPOSITION, AND STRUCTURE AT SIX MONTHS

2.1 Abstract

Background: Understanding the effectiveness of frozen versus fresh osteochondral allografts at moderate post-operative duration (i.e. 6months), and the resultant consequences of traditional freezing, may facilitate *in vivo* maintenance of cartilage integrity. The hypothesis of this study was that the state of the allograft at implantation affects its performance after 6months *in vivo.*

Methods: The effect of frozen versus fresh storage on *in vivo* allograft performance was determined for osteochondral allografts transplanted into seven recipient goats and analyzed at 6months. Allograft performance was assessed by examining osteochondral structure (cartilage thickness, fill, surface location, and surface degeneration, and bone-cartilage interface location), zonal cartilage composition (cellularity, matrix content), and cartilage biomechanical function (stiffness). Relationships between cartilage stiffness versus cartilage composition and surface degeneration were assessed by linear regression.

Results: Fresh allografts maintained cartilage load-bearing function, while also maintaining zonal organization of cartilage cellularity and matrix content, compared to frozen allografts. Overall, allograft performance was similar between fresh allografts and non-operated controls. However, cartilage stiffness was ~80% lower (95%CI, 73-87%) in frozen than non-operated or fresh allografts. Concomitantly, in frozen allografts, matrix content and cellularity were ~55% (95%CI, 22-92%) and ~96% (95%CI, 94-99%) lower, respectively, than non-operated and fresh allografts. Cartilage stiffness correlated positively with cartilage cellularity and matrix content, and negatively with surface degeneration.

Conclusions: Maintenance of cartilage load-bearing function in allografts is associated with zonal maintenance of cartilage cellularity and matrix content. Frozen allografts displayed signs of failure at 6months with cartilage softening, loss of cells and matrix, and/or graft subsidence, supporting the importance of maintaining cell viability during allograft storage, and suggesting that outcomes at 6months may be indicative of long-term (dys)function.

Clinical Relevance: Fresh-vs.-frozen allografts represent the best-vs.-worst conditions with respect to chondrocyte viability, but difficult-vs.-simple with respect to acquisition and distribution. The outcomes described from these two conditions expand the current understanding of *in vivo* cartilage remodeling and describe structural properties (initial graft subsidence), which may have implications for impending graft failure.

2.2 Introduction

The repair of articular defects using osteochondral allografts ideally restores the structure, function, and biology of the cartilage surface. Allograft efficacy may be dependent on sufficient chondrocyte metabolism to preserve cartilage homeostasis and prevent degeneration. Fresh osteochondral allografts are typically implanted with high chondrocyte viability, and retrieved grafts have contained viable chondrocytes up to 29 years after implantation [11, 27, 33, 37, 56]. In contrast, frozen osteochondral allografts, used for massive oncologic joint reconstructions, have low chondrocyte viability at implantation, and grafts retrieved at ~1–5 years contain acellular and often degenerate cartilage [18, 19]. Future improvements in the application of osteochondral allografts would benefit from understanding how the allograft implantation state affects defect repair efficacy and the mechanisms of cartilage maintenance with the best (fresh) and worst (frozen) conditions of cartilage within allografts.

Animal models allow for a systematic and temporal evaluation of cartilage repair and remodeling. Data obtained from human allograft retrievals is skewed toward poor outcomes, since analysis is performed on "failed" cases, where revision or conversion to total joint arthroplasty was required [28, 38, 56]. Clinical evaluations based on patient satisfaction and often limited by availability for long-term follow-up, are not sensitive to early stage degeneration. Intra-disciplinary and multi-scale analyses of cartilage repair in animal models may elucidate tissue changes indicative of early stage degeneration, and provide insight into the time-course of cartilage remodeling and/or deterioration.

Results from such animal models suggest that post-operative time is an important determinant of the quality of cartilage repair. Frozen allografts appear grossly normal, and similar to fresh allografts at short post-operative durations up to

3months [20, 26, 32, 48], and then appear discolored and roughened, often exposing subchondral bone, at 11-12months [26, 45, 48, 51]. In contrast, cartilage of fresh allografts remains grossly normal up to 12months, with only mild degenerative changes [42, 45, 51]. Gross observations are paralleled by histopathologic changes, including reduced (for frozen) and maintained (for fresh) Safranin-O staining intensity [3, 20, 25, 26, 31, 32, 39, 42, 45, 46, 48, 51]. Cartilage cellularity and load-bearing function have been analyzed in relatively few studies. Qualitatively, frozen allografts appear acellular by 3months, compared to fresh allografts, which contain viable cells up to 12months [26, 42, 45, 48, 51]. Confined compressive stiffness was slightly reduced with fresh allografts compared to non-operated controls, and markedly reduced with frozen allografts with increasing post-operative duration [25, 26, 39, 45, 48]. The reduction of cartilage load-bearing function was associated with proteoglycan content [45]; however, other determinants of cartilage stiffness have not been investigated. Rapid indentation of cartilage, which is sensitive to structural measures of cartilage degeneration [4] may be useful to elucidate mechanisms of allograft deterioration or maintenance. Large animal studies with frozen versus fresh allografts at moderate time points (between 3-11months) are needed to understand the progression of cartilage deterioration.

Thus, the hypothesis of this study was that the state of the allograft at implantation affects its performance after 6months *in vivo*. The specific aim was to determine the effect of allograft treatment (frozen versus fresh) on the properties of articular cartilage from retrieved osteochondral allografts at 6months in the goat model, assessing cartilage function, zonal composition, and structure. The results of this study provide a systematic approach and detailed baseline analysis at 6months from which to evaluate *in vivo* cartilage restoration.

2.3   Materials and Methods

Experimental Design

Adult Boer goats (n=8, 2-3yo) were operated (OP) in one knee with IACUC approval. Each OP knee received one FROZEN and one FRESH site-matched osteochondral allograft implanted into alternating medial femoral condyle (MFC) and lateral trochlea (LT) sites (Fig. 2.1A-B). Contralateral knees were Non-Operated (Non-OP) controls. At 6months, animals were euthanized and both knees analyzed (Fig. 2.1C), assessing cartilage function, composition, and structure (Fig. 2.1D).

Donor Preparations

FROZEN and FRESH (each, n=8) donor osteochondral allografts were prepared from both knees of adult Boer goats (n=5, 2-4yo). Hind limbs were received on wet ice within 24hours of sacrifice. Under aseptic conditions, each knee was harvested and divided into condyle and trochlea fragments. Fragments were thoroughly rinsed with phosphate-buffered saline (PBS) supplemented with antibiotics-antimycotics (100U/mL penicillin, 100µg/mL streptomycin, and 0.25µg/mL fungizone, PSF). Some fragments were stored FROZEN at -70°C for 10days. Other fragments were stored FRESH at 4°C for 3days in tissue culture medium (low-glucose Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 0.1mM non-essential amino acids, 2mM L-glutamine, 25µg/mL L-ascorbic acid, and PSF). Following implantation, chondrocyte viability near the graft site was assessed with LIVE/DEAD® (Molecular Probes, Eugene, OR) staining and fluorescent microscopy,[40] and was low (<10%) for FROZEN, and high (>90%) for FRESH grafts.

Allograft Surgery

Osteochondral allografting was performed through a medial knee arthrotomy under general anesthesia. General anesthesia was induced intravenously with diazepam (0.22mg/kg) and ketamine (10mg/kg), and maintained with isoflurane inhalation (0.4-3%). In addition, cefazolin (1g) was given intravenously prior to incision and just after closure. The knee joint was exposed through a medial parapatellar incision, and lateral patella dislocation. An osteochondral defect (d=7.5mm, h=5mm) was created at MFC and LT sites, using a guide pin and surgical reamer (Arthrex, Inc., Naples, FL) under continuous saline irrigation (Fig. 2.2A). An orthotopic cylindrical osteochondral plug (d=8mm, h=5mm) was harvested from donor tissue using a coring reamer (Arthrex, Inc., Naples, FL), thoroughly cleansed with saline, and inserted carefully into the defect with impaction using a graft tamp and mallet (mean±SD, 35±15 taps, each with 161±46N for 2.93±3.96 ms duration and 0.22±0.27N•s impulse) [7] until the graft was flush with the surrounding articular surface (Fig. 2.2B). Following closure, OP knees were cast in a modified Thomas-Schroeder splint for 13days to limit weight-bearing.

Animals were monitored post-operatively for pain and lameness throughout the study. One day post-operation, animals received intramuscular injections of long-acting antibiotic, Combi-Pen-48 (900,000U penicillin G), pain medication/narcotic (0.6mg buprenorphine), and non-steroidal anti-inflammatory (150mg ketoprofen). Pain management also included a fentanyl patch (10.2mg released over 72hours). Joint inflammation and lameness were monitored until animals were sound. After 6months, animals were euthanized with potassium chloride (1-2mmol/kg) intravenously under stage III anesthesia. One animal died of a suspected upper respiratory infection 10days post-operation, unrelated to the implant, and analysis was not included in the study.

Repair Site Analysis

Intact knee joints were received on wet ice within 24hours of sacrifice. Distal femurs were harvested, photographed, and examined grossly for cartilage fill and integration. Joints were tested biomechanically, and then samples were divided, as detailed in the §2.7 Appendix, and analyzed for chondrocyte biosynthesis, cellularity, matrix content, and osteochondral structure.

Biomechanical Function

Cartilage load-bearing function was assessed by indentation testing at the center of each osteochondral core. Using a benchtop mechanical tester (v500cs, BioSyntech Canada Inc), samples were compressed rapidly by 100μm, at three sites 0.5mm apart (proximal to distal) [34]. The peak load was divided by the indentation depth, normalized to cartilage thickness and indenter tip area, and three points averaged to determine the indentation material stiffness, expressed in units of MPa.

Cartilage Composition

Cellularity was assessed with depth from the articular surface. Cells were labeled fluorescently, and full-thickness cartilage was imaged along a vertical profile to a depth of 45μm at 15μm intervals. Image stacks were processed with a custom routine to localize and count cells [40]. Cellularity was calculated as the number of cells divided by cartilage volume (cells/cm^3) for superficial, middle, and deep zones of cartilage, defined as 0-15%, 15-50%, and 50-100% of the cartilage thickness.

Matrix fixed charge in cartilage was assessed by Hexabrix-Enhanced micro-computed tomography (HE-μCT). Hexabrix, an ionic contrast agent, distributes

inversely to the fixed charge density in soft tissues, and is therefore a sensitive (inverse) indicator of proteoglycan content [41]. HE-μCT Gray-Value (i.e. x-ray attenuation) was calculated, as described in the §2.7 Appendix, in the superficial, middle, and deep zones were defined as the top 90μm, the next 35%, and the remaining ~50% of the cartilage thickness, respectively. The spatial variation in matrix fixed charge was illustrated with color maps representing 5%-95% HE-μCT Gray-Value within the cartilage.

Osteochondral Structure

Cartilage and bone structure were assessed by μCT, quantifying cartilage thickness, fill, and surface location, and bone-cartilage interface location, evaluated within a 2.5mm radius of the repair center (see §2.7 Appendix). The repair center was determined as the midpoint between visually identified discontinuities in the bone, or the corresponding Non-OP location.

Cartilage histopathology was assessed with the modified Mankin score [49] using Safranin-O and Hematoxylin/Eosin (H&E) stained slides because osteochondral allograft repair resembles normal hyaline cartilage structure at the time of implant. The Mankin scale is sensitive to early stage degeneration and was previously found to correlate with indentation stiffness [4]. The histopathology score had a 0-15 scale, with high scores corresponding to degeneration. Graft-host bone integration was assessed by binary histological evaluation of immune response (cellular infiltrate and/or pannus), fibrous marrow, subchondral cysts, and bone healing.

Statistical Analysis

Data are presented as mean±SD. The effect of allograft treatment (Non-OP, FROZEN, FRESH) and site (MFC, LT) on cartilage cellularity, stiffness, thickness, HE-μCT Gray-Value, structural parameters were determined by 2-way ANOVA. For cellularity and HE-μCT Gray-Value data, zone (superficial, middle, deep) was considered a repeated measure, and a three-way repeated measures ANOVA was performed. At each site, Tukey post-hoc comparisons were performed to compare treatments with significant differences ($p < 0.05$). For nonparametric data (i.e. histopathology scores), samples were analyzed analogously using Friedman and Dunn's tests. Power analysis ($\alpha = 0.05$ and $1 - \beta = 80\%$) was performed with cartilage indentation stiffness as primary endpoint, and n=4 samples per treatment group was determined to detect a difference of 150% of the SD, an effect size sufficient to distinguish microfracture repair from non-operated cartilage in goats at 6months [29]. For secondary endpoints where effect size may be smaller, power may not be sufficient to detect significant differences.

The relationship between allograft performance versus cartilage composition and surface roughness/degeneration were assessed by linear regression. Biomechanical function (stiffness) was correlated with cartilage cellularity, and relative matrix fixed charge. Biomechanical function was also correlated with histopathology, and surface irregularity by nonparametric Spearman's rank method [24]. Coefficients of determination, R^2 (parametric) and ρ^2 (nonparametric), are reported for significant relationships ($p < 0.05$).

2.4   Results

Biomechanical Function

Cartilage material stiffness varied with allograft treatment (p<0.001), but not with site (p=0.3), whereas cartilage thickness varied with site (p<0.001), but was not affected by treatment (p=0.3). Stiffness in Non-OP was similar to FRESH (p>0.9). Stiffness was ~80% lower in FROZEN than Non-OP (95% Confidence Interval [CI], 79-84%) and FRESH (95%CI, 73-87%) allografts, and was 11.5±4.4MPa at MFC and 14.2±5.3 at LT in Non-OP, but only 2.0±0.8MPa at MFC (p<0.05 vs. Non-OP, p=0.1 vs. FRESH, Fig. 2.3A) and 2.7±0.5MPa at LT (p<0.01 vs. Non-OP, p<0.05 vs. FRESH, Fig. 2.3B) in FROZEN. Cartilage thickness was ~60% lower (95%CI, 52-68%) at LT (0.86±0.23mm) than at MFC (1.44±0.34mm, Fig. 2.3C-D).

Cartilage Composition

Chondrocyte cellularity varied with allograft treatment (p<0.001), but not with site (p=0.2). Cellularity was ~96% lower in FROZEN than Non-OP (95%CI, 94-98%) and FRESH (95%CI, 94-99%) allografts at MFC (each, p<0.001, Fig. 2.4A), and LT (each, p<0.01, Fig. 2.4B). Throughout the cartilage depth, cellularity varied by zone (p<0.001), with significant interactions between zone and treatment (p<0.05) and zone and site (p<0.05). At MFC, cellularity in Non-OP decreased with depth from the articular surface, and was $38.6±19.6x10^6$cells/cm^3 in superficial, $18.4±6.9x10^6$cells/cm^3 in middle, and $13.6±6.0x10^6$cells/cm^3 in deep zones, and was similar to FRESH (p>0.5). At LT, cellularity in Non-OP did not vary significantly with depth (p=0.1), and was $39.0±7.6x10^6$, $31.4±13.8x10^6$, and $28.6±13.3x10^6$cells/cm^3 in superficial, middle, and deep zones, respectively, and was similar to cellularity in FRESH allografts for each zone (each, p=1.0).

Collectively, HE-μCT analysis and Safranin-O histology indicated that matrix fixed charge was similar between Non-OP and FRESH, and was lower in FROZEN allografts. HE-μCT Gray-Value for Non-OP and FRESH demonstrated the normal spatial variation of matrix fixed charge, which increases with depth from the articular surface. High HE-μCT Gray-Value (in red and yellow), which represents low matrix fixed charge, was present throughout the depth of FROZEN (Fig. 2.5B,E), and only at the articular surface of Non-OP (Fig. 2.5A,D) and FRESH (Fig. 2.5C,F). Low HE-μCT Gray-Value (in blue), which represents high matrix fixed charge, was absent in FROZEN, and was present in the deep zone of Non-OP and FRESH. Representative Safranin-O histology confirmed the extensive loss of proteoglycans in FROZEN versus Non-Op and FRESH allografts (Fig. 2.5G-L). Safranin-O score in Non-OP was similar to FRESH ($p > 0.3$). Safranin-O score was 3.0 ± 0.0, indicating absence of staining, in FROZEN, and higher than Non-Op and FRESH at MFC (each, $p < 0.05$) and LT (each, $p < 0.001$, Table 2.1).

Quantitatively, in addition to visualizing overall qualitative patterns, HE-μCT delineated the spatial variation of matrix fixed charge throughout the cartilage depth. HE-μCT Gray-Value varied with cartilage zone ($p < 0.001$), but not with allograft treatment ($p = 0.4$), and with a significant interaction between zone and treatment ($p < 0.05$). HE-μCT Gray-Value in Non-OP was similar to FRESH at MFC and LT (each, $p > 0.5$). In the deep zone, matrix fixed charge was lower in FROZEN, as indicated by ~55% increase in HE-μCT Gray-Value at MFC (each, $p < 0.01$, Fig. 2.6A) and LT ($p < 0.05$ vs. Non-OP, $p = 0.06$ vs. FRESH, Fig. 2.6B) than Non-OP (95%CI, 22-67%) and FRESH (95%CI, 39-92%).

Osteochondral Structure

Cartilage and bone structure, measured by cartilage fill and surface location, and bone-cartilage interface location, varied with allograft treatment (p<0.001). At MFC, cartilage fill was ~55% lower (95%CI, 34-100%) in FROZEN than FRESH (p<0.05, Fig. 2.7A), in association with -1.12 ± 0.94mm and -1.46 ± 0.88mm depression of the cartilage surface and bone-cartilage interface (each, p<0.05, Fig. 2.7C,E). At LT, cartilage fill tended to be lower, by ~20% (95%CI, 3-55%), in FROZEN than FRESH (p=0.1, Fig. 2.7B); the articular surface tended to be depressed by -0.30 ± 0.07mm (p=0.1, Fig. 2.7D), and the bone-cartilage interface was depressed by -0.48 ± 0.29mm (p<0.05, Fig. 2.7F).

Gross macroscopic observations of allografts were visually consistent with the quantified structural parameters. Overall joints appeared macroscopically normal, with no osteophyte formation or extensive degeneration (Fig. 2.8A-B). Implanted allografts remained clearly demarcated from the surrounding host cartilage at retrieval. At MFC, the cartilage surface of FROZEN allografts was visually sunk with respect to the surrounding host cartilage (2/3 allografts, Fig. 2.8C-2,3), whereas the cartilage surface of FRESH allografts was smooth (2/4, Fig. 2.8D-4,5) or appeared flush to the surrounding host cartilage with minimal uneven surfaces (2/4, Fig. 2.8D-6,7). At LT, the cartilage surface of FROZEN allografts appeared flush to the surrounding host cartilage with visible fibrous tissue or degeneration at the graft margins (4/4 allografts, Fig. 2.8E-2,4,5,7), whereas the cartilage surface of FRESH allografts was smooth (2/3, Fig. 2.8F-3,6).

Histopathology and surface irregularity scores varied with allograft treatment (each, p<0.001), site (each, p<0.01), and with significant interaction between treatment and site (each, p<0.05). Histopathology score was similar between Non-Op and FRESH, and was higher, corresponding to more degeneration, for FROZEN

(Table 2.1). At MFC, Non-Op histopathology score was 2.1±1.9, which was lower than FROZEN (p<0.05), and similar to FRESH (p=0.8), but surface irregularity score did not vary among treatments (p>0.6), and was 0.9±0.5 for all MFC samples. At LT, Non-Op histopathology score was 0.4±0.5, which was lower than FROZEN (p<0.001), and similar to FRESH (p=0.3). Concomitantly, surface irregularity scores were indistinguishable between Non-Op and FRESH (p=1.0, Table 2.1), but higher for FROZEN than Non-OP and FRESH (each, p<0.001).

All FROZEN and FRESH allografts exhibited bony union at the graft-host junction, but were variably accompanied by structural abnormalities of bone healing (Fig. 2.9). At MFC, bone marrow exhibited fibrosis (2/3 FROZEN, 2/4 FRESH), and cellular infiltrates (2/3 FROZEN, 1/4 FRESH). Bone marrow histology was similar at LT; marrow was fibrotic in FROZEN (3/4) and FRESH (2/3), and was accompanied by occasional cellular infiltrates (each, 1 allograft). In contrast, subchondral cysts were widespread at MFC for both FROZEN (2/3) and FRESH (4/4), but less common at LT (each, 1 allograft).

Determinants of Allograft Performance

Biomechanical properties correlated significantly with cartilage cellularity, matrix content, and surface degeneration. For MFC+LT, cartilage stiffness correlated positively with overall cellularity (R^2=0.46, p<0.001, Fig. 2.10A), and negatively with deep zone HE-μCT Gray-Value (R^2=0.23, p<0.05, Fig. 2.10B), histopathology score (ρ^2=0.75, p<0.001, Fig. 2.10C) and surface irregularity score (ρ^2=0.52, p<0.01, Fig. 2.10D). MFC and LT also exhibited similar trends to MFC+LT, except cartilage stiffness versus surface irregularity for MFC (ρ^2=0.25, p=0.4, Fig. 2.10D).

2.5   Discussion

These results demonstrate marked differences in cartilage zonal composition, structure, and function of osteochondral allografts depending on their FRESH versus FROZEN treatment, as early as 6months post-repair. The cartilage of FROZEN osteochondral allografts was mechanically soft (Fig. 2.3), acellular (Fig. 2.4), depleted of matrix fixed charge/proteoglycans (Figs. 2.5B,E,H,K, 2.6), and visually deteriorated (Fig. 2.8), suggesting that storage conditions that eliminate chondrocyte viability at implantation also reduce allograft performance *in vivo*. In contrast, the cartilage of FRESH osteochondral allografts had similar mechanical properties (Fig. 2.3), and cellular (Fig. 2.4), and matrix (Figs. 2.5C,F,I,L, 2.6) zonal organization to Non-OP site-matched cartilage (Figs. 2.3, 2.4, 2.5A,D,G,J, 2.6), and appeared smooth and flush to the surrounding host cartilage (Fig. 2.8). Maintenance of cartilage load-bearing function was associated with zonal maintenance of chondrocyte cellularity and matrix fixed charge. Thus, the *in vivo* performance of osteochondral allografts at 6months paralleled the chondrocyte viability within the allograft at the time of implantation.

The use of the goat model to study cartilage restoration by osteochondral allografting involved the consideration of a number of issues. Two experimental sites (MFC and LT) within each joint doubled the examined sample number, while also providing similar overall joint environments for FROZEN versus FRESH treatment groups. Alternating treatments between sites avoided possible effects specific to one site. Even with the limited sample numbers and some variation in gross observations within treatment groups, there were clear differences in some parameters (stiffness, cellularity, matrix content) irrespective of site, and differences in other parameters (osteochondral structure) that were site-dependent.

While the general consensus of the literature suggests that fresh grafts perform "better" than frozen grafts, this study expands upon such generalizations and introduces three new key findings. First, by 6months, the cartilage of frozen osteochondral allografts exhibited failure based on softening and associated deterioration of structural indices, particularly cellularity, deep zone fixed charge, and surface irregularity. Second, the cartilage of fresh osteochondral allografts maintained its zonal distribution of cellular and matrix components and load-bearing function similar to non-operated cartilage. Third, allografts failed differently at MFC and LT sites, with graft subsidence at MFC, but not at LT, suggesting that repair efficacy is site- and possibly loading-dependent.

At 6months, FROZEN allografts already exhibited clear progression toward failure, with loss of chondrocytes, reduced proteoglycan content and cartilage stiffness, and associated surface and/or bone collapse at MFC. Reduced cellularity (Fig. 2.4) and proteoglycan synthesis rates (§2.7 Appendix) suggest inadequate biological remodeling capacity and the inability to sustain long-term function. End-stage failure of FROZEN allografts, accompanied here by loss of zonal architecture, increased surface irregularity, and reduced stiffness and proteoglycan content (Figs. 2.3-2.8), was consistent with previous long-term animal studies [26, 45, 48, 51] and with grafts retrieved after limb-salvaging oncologic reconstructive surgery [18, 19]. Hence, 6months was a sufficient time for grafts to fail at MFC and for grafts to exhibit progressive deterioration toward failure at LT.

In contrast, FRESH allografts preserved depth-dependent tissue properties similar to non-operated cartilage, and thus maintained their capacity for biological homeostasis. Even in mechanically demanding environments (i.e. MFC), FRESH allografts mimicked "normal" zonal organization of cellular and matrix components

present in non-operated cartilage (Figs. 2.4-2.6). Previous allograft animal studies did not analyze depth-related variations in chondrocyte cellularity [31, 45, 51] and matrix content [3, 25, 26, 31, 39, 51], which are important since chondrocytes exhibit zone-specific functions [2]. Appropriate zonal chondrocyte organization and proteoglycan synthesis rates in FRESH allografts provided biological remodeling to preserve the articular surface, sustain bulk tissue properties, including cartilage fill, matrix content, and load-bearing function, maintain the bone-cartilage interface and underlying bone structure, and prevent progressive deterioration to graft collapse (Figs. 2.3, 2.5-2.9). Usage of such fresh allografts has resulted in clinical success rates >75% [6, 8, 10, 12, 17, 23, 36]. However, it remains to be determined the relative importance of cartilage zone(s) to preserve depth-dependent tissue homeostasis and prevent failure progression.

The different effects of allograft treatment on osteochondral structure at MFC and LT suggests that repair efficacy at mechanically demanding sites is more sensitive to decreased implant viability. In a goat model, spontaneous repair of cartilage defects at LT had better histological scores and gross visual appearance than at MFC after 6 and 12weeks *in vivo* [15]. In the present 6month study, FROZEN allografts had reduced cartilage fill and increased surface degeneration at MFC but not at LT, which may be due to inherent differences between anatomic sites, with cartilage thickness and loading environment likely contributing to the variation. Thus, higher chondrocyte viability at implant may be needed to sustain biological remodeling at mechanically demanding sites, such as the MFC.

Allograft performance may also be related to effective osseous integration. Fresh allografts retrievals often demonstrated that graft failure was associated with limited osseous incorporation, graft collapse, osteonecrosis, and pannus formation

[56]. Such osseous integration, influenced, in part, by the immunogenicity of allogenic bone, has been improved by reducing surface antigens (by freezing) [21, 47] or matching leukocyte-antigens, with accompanying improvement in clinical outcomes [22, 50]. While subchondral bone appeared generally maintained, there was histopathological evidence of bone abnormalities (i.e. fibrotic marrow, cysts) for both frozen and fresh allografts in the present study. Antigen matching was not attempted, in accordance with current clinical practice, but may be a contributing factor. Further investigation of the relationship between osseous remodeling and cartilage maintenance is needed.

This systematic approach used to evaluate cartilage restoration by frozen and fresh allografts, could also be applied to the efficacy of other cartilage repair therapies, tissue-engineered cartilaginous/osteocartilaginous grafts, and stored osteochondral grafts. Chondrocytes, in grafts stored routinely at 4°C to accommodate regulatory screening for infectious diseases [1], are susceptible, with increasing storage duration, to cell death especially at the articular surface [5, 13, 16, 43, 44, 52-54, 57]. Usage of 4°C stored allografts, leads to some short-term clinical improvement compared to pre-operative patient function [14, 30, 35, 55]. The long-term *in vivo* performance of 4°C stored allografts, which are used after prolonged storage durations (range 10-43days) [14, 30, 35, 55] and represent intermediate condition of cellularity, are unknown. Further analysis of zonal chondrocyte organization following cartilage repair treatments may help to elucidate a critical number of functional chondrocytes in the appropriate cartilage zone(s) to maintain the surrounding matrix, and subsequent biomechanical function of the allograft *in vivo*.

2.6   Acknowledgments

Table 2.1: Histopathology and cartilage degeneration scores. [a]Histopathology score (0-15) was determined by assigning a score of 0-2 or 0-3 (with 0 representing normal, and 2 or 3 representing severe degeneration) for the following histologic characteristics: surface irregularity (0-2), vertical clefts into the transitional or radial zone (0-2), transverse clefts (0-2), cloning (0-3), hypocellularity (0-3), and Safranin-O staining (0-3) [49]. [b]Avg Rank was calculated for non-parametric Friedman's test, in which the ordinal value for a sample is replaced by its rank (with ties, from 1-28) when the data are sorted from low to high. $*p<0.05$, $**p<0.001$, $***p<0.001$ vs. FROZEN.

	MFC			LT		
	Non-OP	**FROZEN**	**FRESH**	**Non-OP**	**FROZEN**	**FRESH**
n	7	3	4	7	4	3
Histopathology Score[a] (Avg Rank)[b]	2.1±1.9* (13.9)	8.3±1.5 (25.8)	2.5±1.0 (16.9)	0.4±0.5** (7.5)	7.5±1.0 (24.4)	0.0±0.0*** (4.5)
Safranin-O Score (Avg Rank)[b]	0.6±0.8* (11.9)	3.0±0.0 (25.0)	0.5±0.6* (12.0)	0.4±0.5*** (11.3)	3.0±0.0 (25.0)	0.0±0.0*** (7.0)
Surface Irregularity Score (Avg Rank)[b]	0.7±0.8 (15.8)	1.0±0.0 (20.5)	1.0±0.0 (20.5)	0.0±0.0*** (7.0)	1.0±0.0 (20.5)	0.0±0.0*** (7.0)
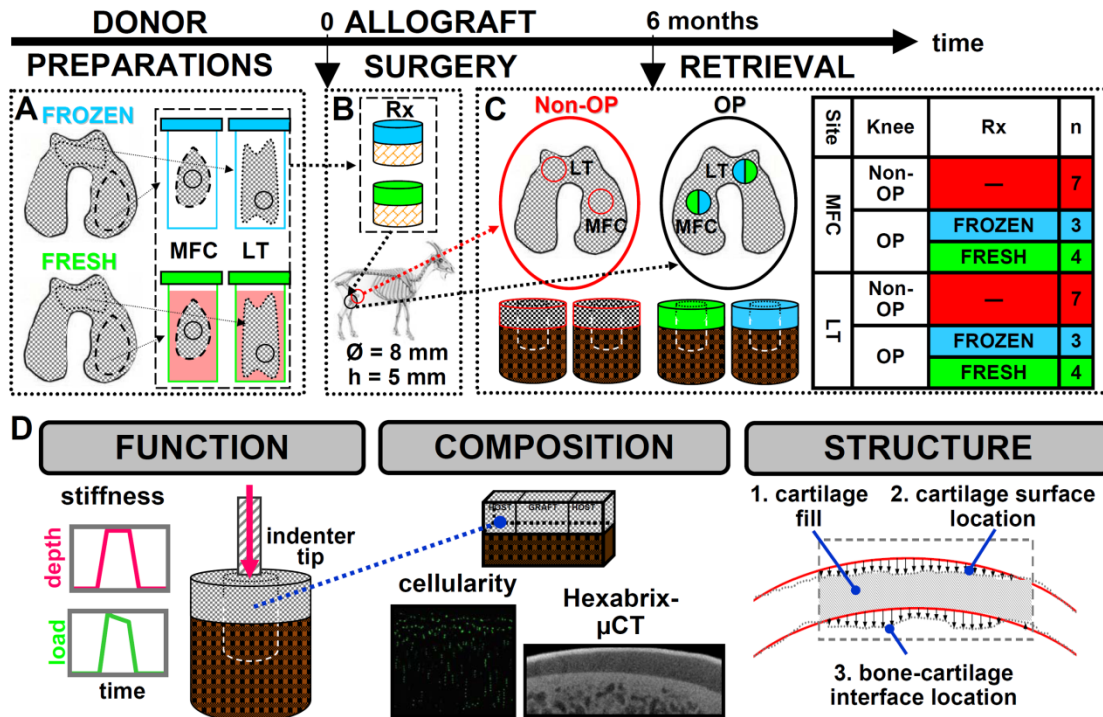
Figure 2.1: Schematic diagrams and timeline for the experimental design, including (**A**) donor preparations, (**B**) allograft surgery, (**C**) retrieval groups, and (**D**) analysis for biomechanical function, cartilage composition, and osteochondral structure.
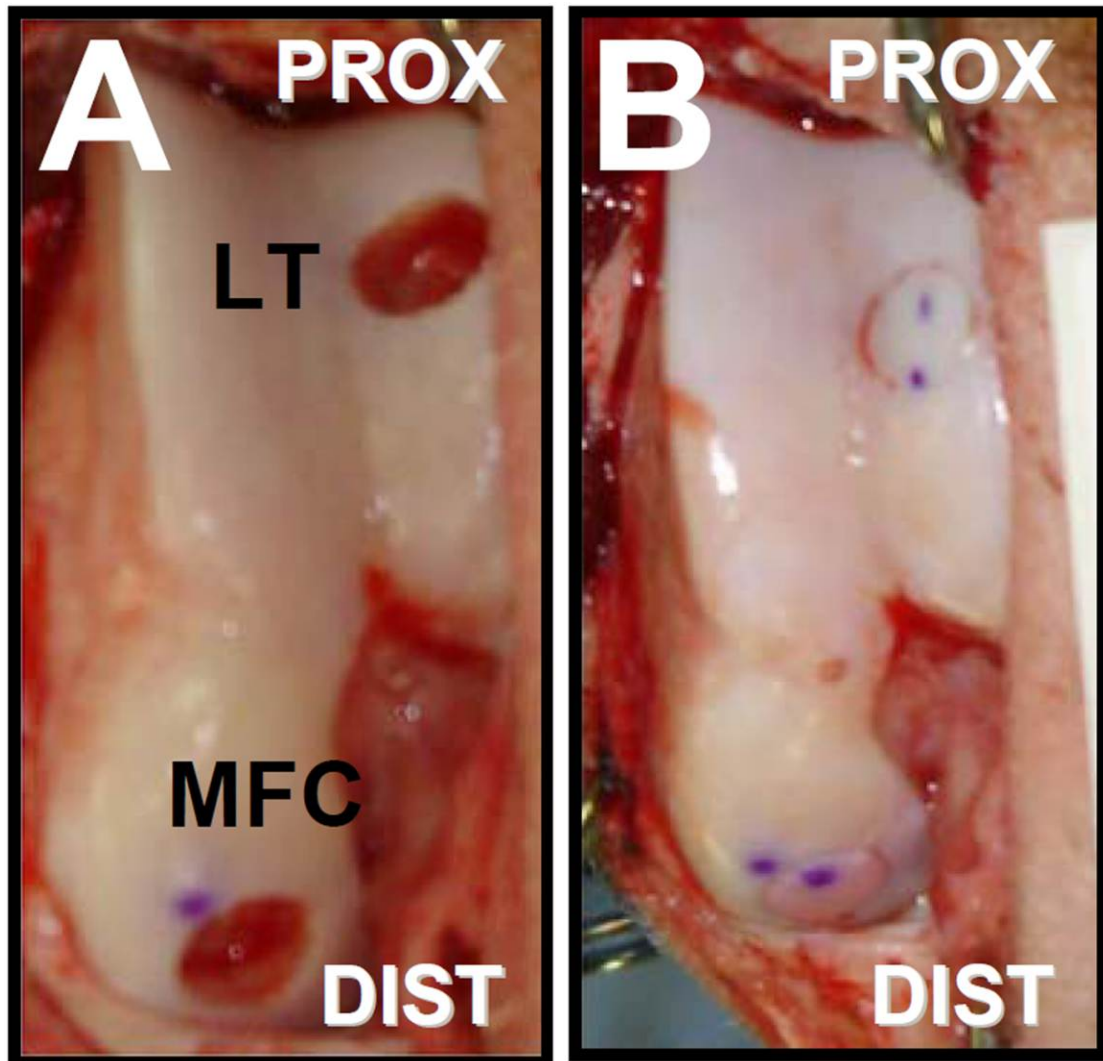
Figure 2.2: Allograft surgical procedure demonstrating the (**A**) osteochondral defects at MFC and LT, and (**B**) implanted allografts at t=0.
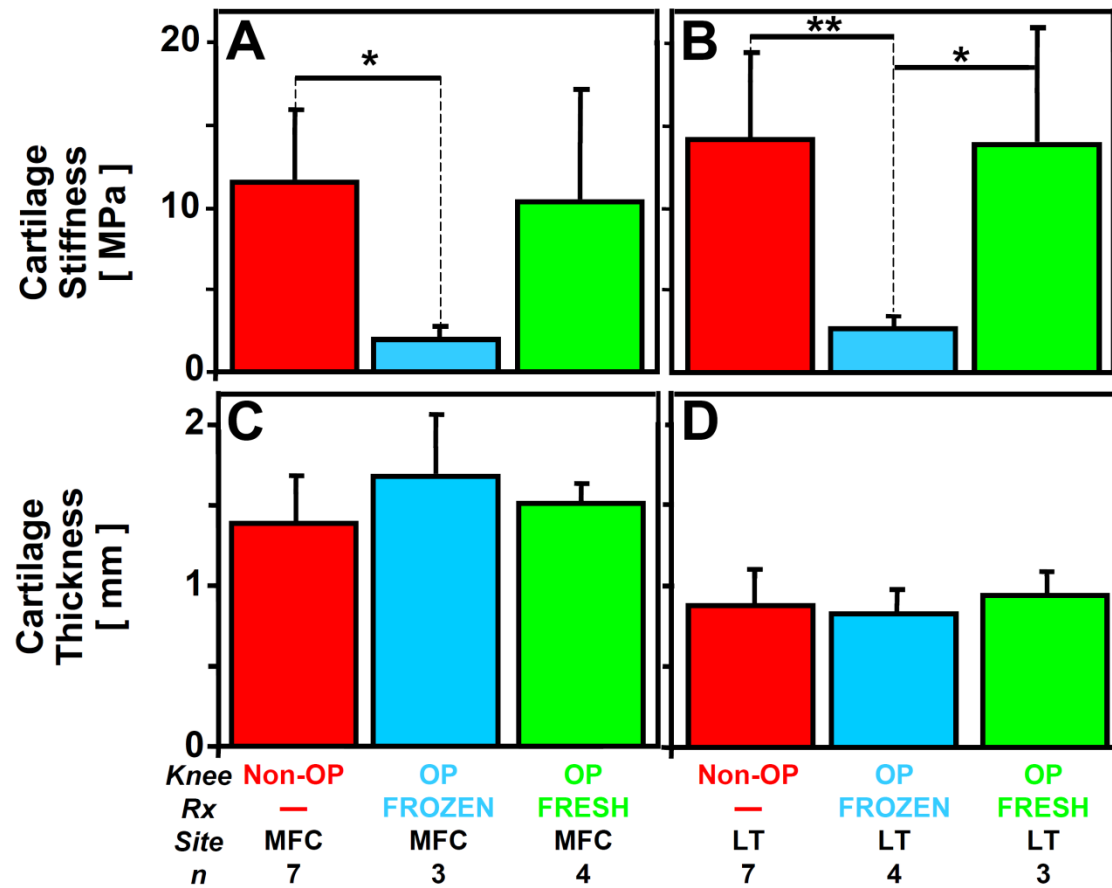
Figure 2.3: Effect of *in vivo* allograft treatment on cartilage (**A,B**) stiffness and (**C,D**) thickness in retrieved osteochondral allografts at (**A,C**) MFC and (**B,D**) LT after 6months.
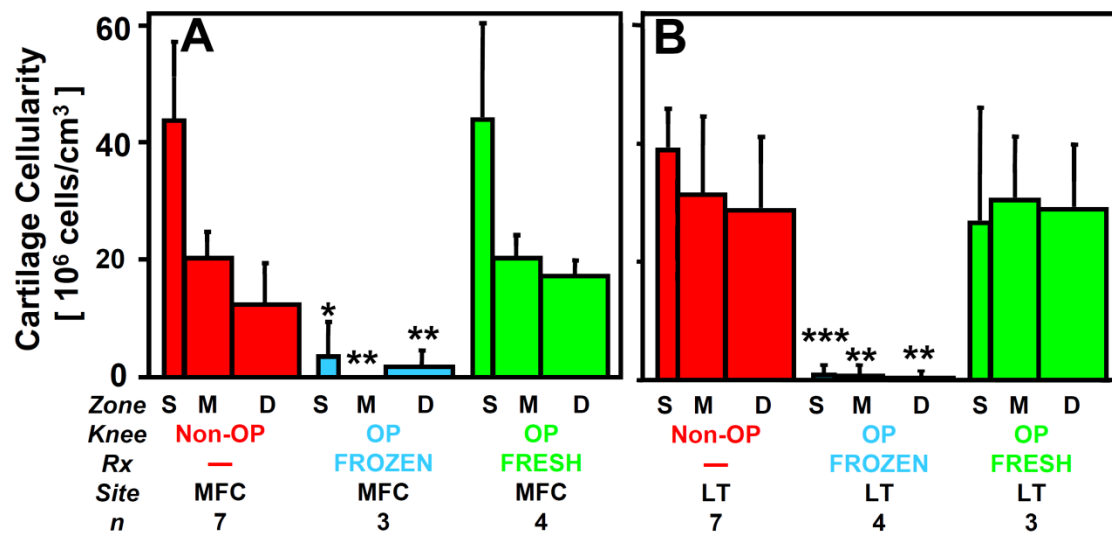
Figure 2.4: Effect of *in vivo* allograft treatment on cartilage cellularity in retrieved osteochondral allografts at (**A**) MFC and (**B**) LT after 6months.
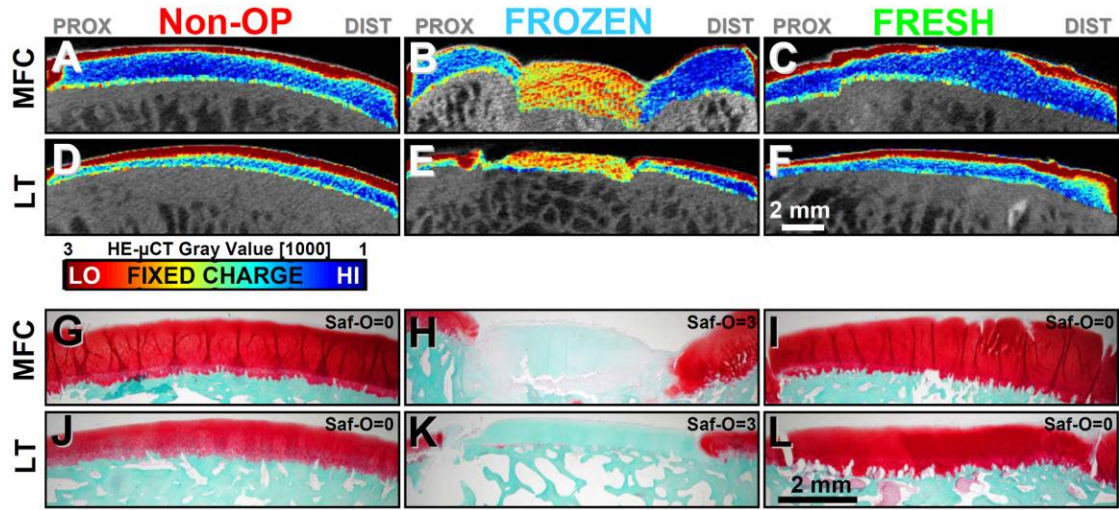
Figure 2.5: Representative (**A-F**) color maps of HE-μCT analysis and corresponding (**G-L**) Safranin-O histology at (**A-C,G-I**) MFC and (**D-F,J-L**) LT of (**A,D,G,J**) Non-OP, (**B,E,H,K**) FROZEN, and (**C,F,I,L**) FRESH retrieved osteochondral allografts after 6months *in vivo*.
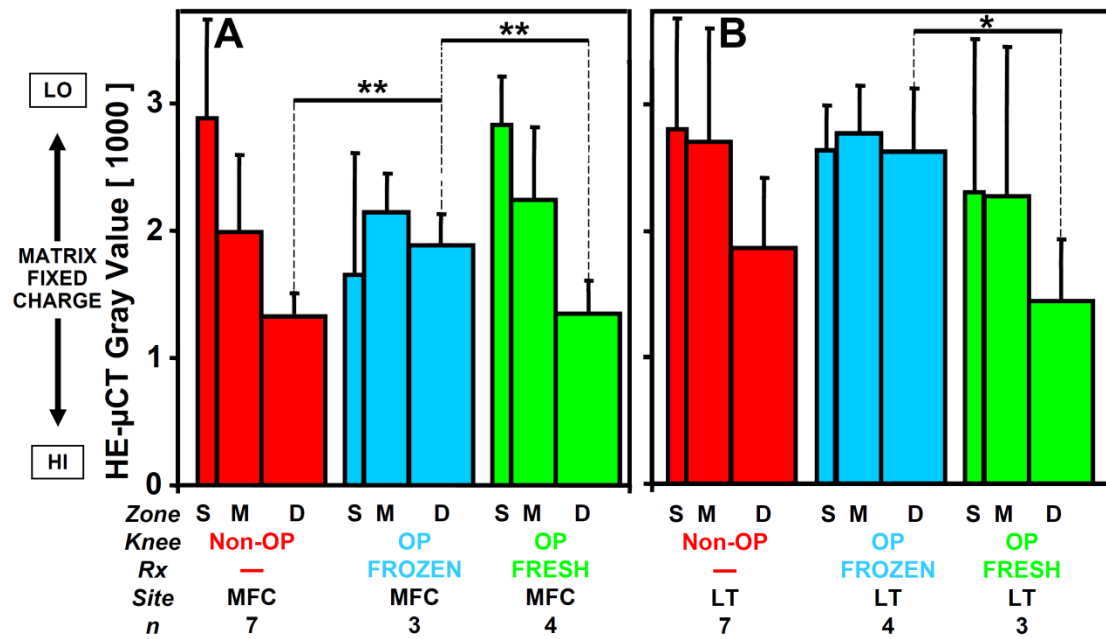
Figure 2.6: Effect of *in vivo* allograft treatment on Hexabrix-Enhanced μCT (HE-μCT) gray value in cartilage of retrieved osteochondral allografts at (**A**) MFC and (**B**) LT after 6months.
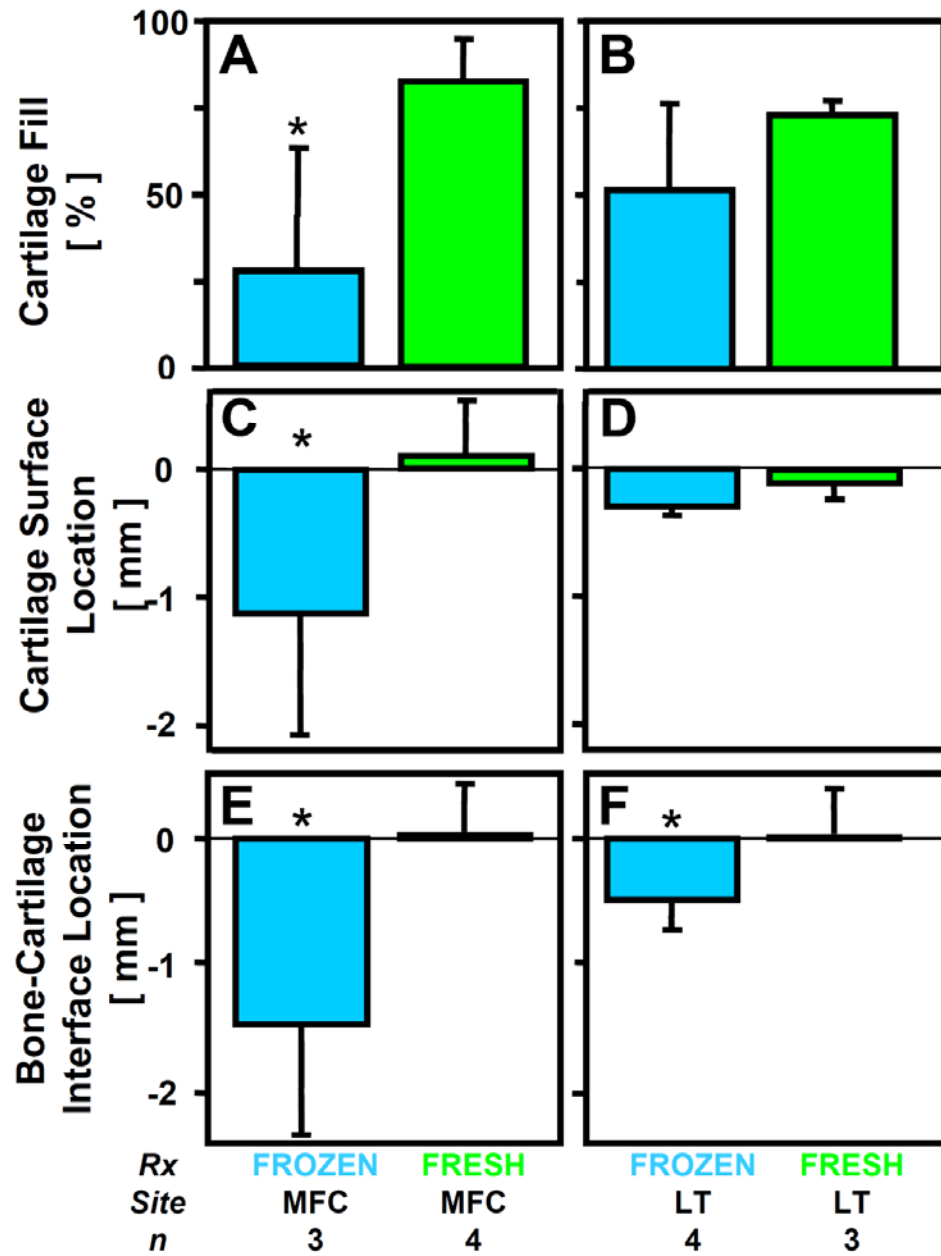
Figure 2.7: Effect of *in vivo* allograft treatment on (**A,B**) cartilage fill, (**C,D**) cartilage surface location, and (**E,F**) bone-cartilage interface location at (**A,C,E**) MFC and (**B,D,F**) LT after 6months.
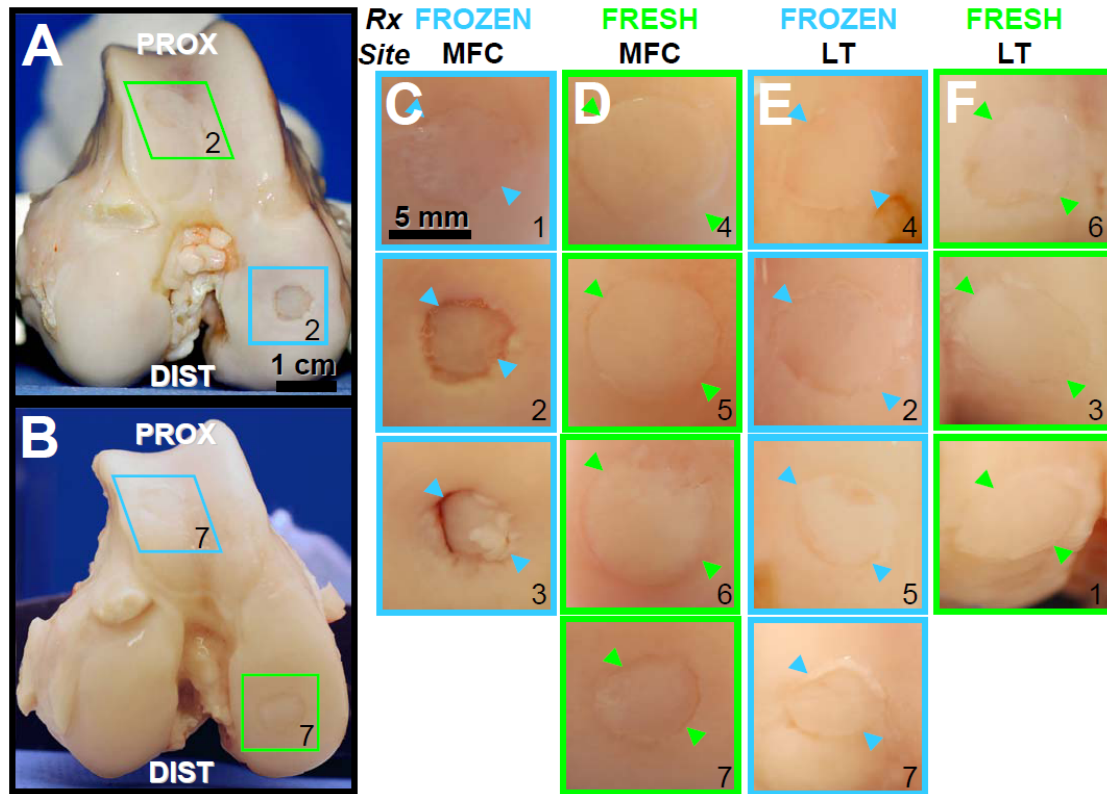
Figure 2.8: Gross macroscopic images of (**A,B**) representative knee joints after 6 months *in vivo*, and individual retrieved FROZEN (blue) and FRESH (green) osteochondral allografts at (**C,D**) MFC and (**E,F**) LT. Numbers in lower right corner indicate the recipient animal number. Arrowheads indicate borders of the allograft and host cartilage.
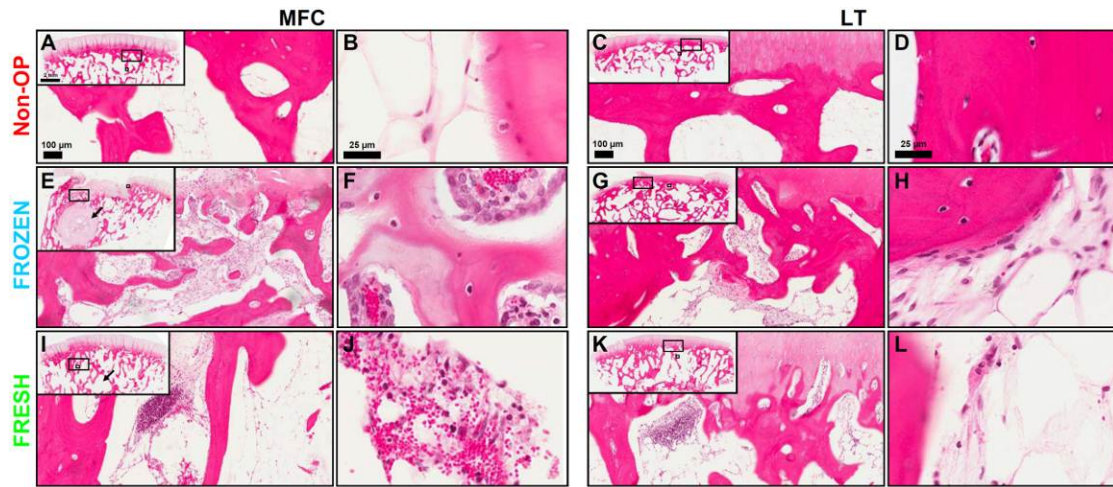
Figure 2.9: Representative H&E bone histology for (**A-D**) Non-OP, (**E-H**) FROZEN, and (**I-L**) FRESH allografts at (**A,B,E,F,I,J**) MFC and (**C,D,G,H,K,L**) LT. (**A-D**) Bone marrow contained primarily fat cells. (**E-L**) Bone marrow was fibrotic, containing spindle-shaped purple fibroblasts in addition to fat cells. (**E-I**) Bone cysts indicated by arrows; bone cysts were visually confirmed by gross evaluation (either as void spaces in bone or containing gelatinous-like material). (**F,J**) Cellular infiltrate was noted as clusters of small, round pink eosinophils.
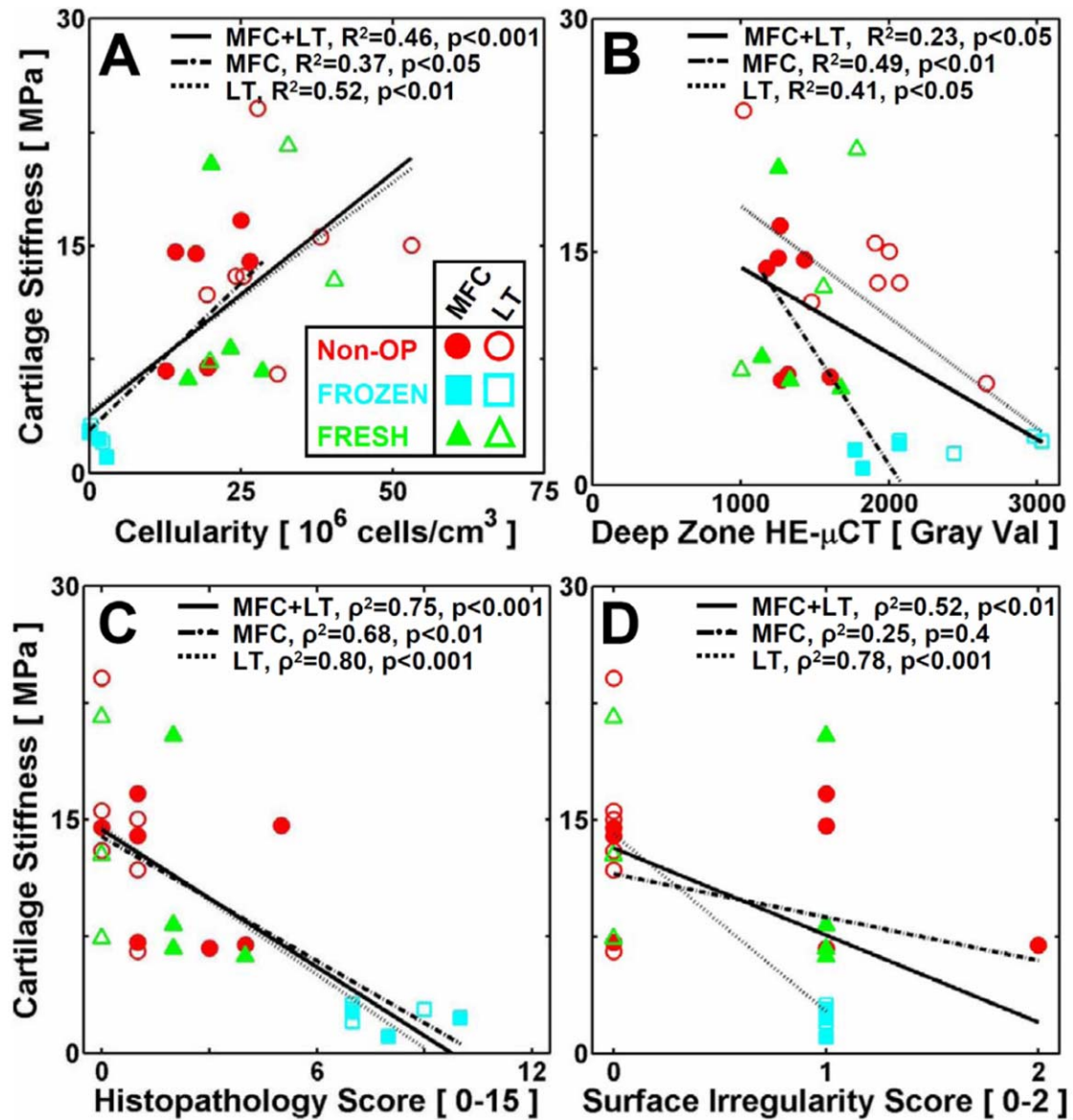
Figure 2.10: **(A,B)** Parametric regression analysis and **(C,D)** Spearman's rank correlation of cartilage stiffness versus **(A)** cellularity, **(B)** HE-μCT Gray-Value in the deep zone, **(C)** histopathology score, and **(D)** surface irregularity score for MFC (filled) and LT (open). Data points correspond to individual Non-OP (red circles), FROZEN (blue squares), and FRESH (green triangles) allograft retrievals. Significance (p) and correlation coefficients (R^2 for **A-B**, ρ^2 for **C-D**) were determined. Lines represent the linear regression fits of the data and are shown only to indicate trends.

2.7   Appendix

Methods

Tissue Division for Repair Site Analysis

Intact knee joints were received on wet ice within 24hours of sacrifice. Distal femurs were harvested, photographed, and examined grossly for cartilage fill and integration. Next, osteochondral cores (d=15mm, h~8-10mm) were isolated to encompass the experimental site, using a custom coring bit under irrigation with cold PBS+PSF. Following biomechanical testing, osteochondral slabs (width=2mm), cut centrally from proximal to distal, were isolated. Some of the remaining cartilage, corresponding to ~1/4 defect repair, was removed from the bone and analyzed for chondrocyte biosynthesis. Osteochondral slabs were analyzed for cellularity, subsequently fixed in 10% neutral buffered formalin containing 1% cetylpyridinium chloride, and analyzed for matrix content and osteochondral structure.

Cartilage Metabolism

Chondrocyte glycosaminoglycan biosynthesis was assessed as ^{35}S-sulfate-incorporation. For samples with sufficient tissue (Non-Op=8, FROZEN=2, FRESH=4), full-thickness cartilage was incubated in tissue culture medium (low-glucose Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and PSF) containing 5μCi/mL ^{35}S-sodium sulfate (Perkin Elmer Life Sciences, Boston, MA) for 48hours at 37°C in 5%CO_2/95%O_2 humidified incubator. ^{35}S-sulfate-incorporation was expressed as counts per minute/mg dry weight [CPM/mg] [56].

Matrix Fixed Charge by HE-μCT

Matrix fixed charge in cartilage was assessed by Hexabrix-Enhanced micro-computed tomography (HE-μCT). Hexabrix, an ionic contrast agent, distributes inversely to the fixed charge density in soft tissues, and is therefore a sensitive (inverse) indicator of proteoglycan content [41]. Fixed osteochondral slabs were incubated for ~1day in a Hexabrix (20%, by volume in PBS) solution, and then imaged in 3D using μCT (GE eXplore Locus): 80 kV, voxel=(45 μm)^3. In a sagittal slice, including the indentation site, from 3D μCT datasets, cartilage and bone contours were determined by applying thresholds [9]. HE-μCT Gray-Value (i.e. x-ray attenuation) was calculated within a region of segmented cartilage (width=2mm) surrounding the indentation site for the overall cartilage thickness, as well as zones of cartilage, excluding two pixels at the articular cartilage surface and bone interface to minimize partial volume effects; superficial, middle, and deep zones were defined as the top 90μm, the next 35%, and the remaining ~50% of the cartilage thickness, respectively. The spatial variation in matrix fixed charge was illustrated with color maps representing 5%-95% HE-μCT Gray-Value within the cartilage.

μCT Structural Properties

Cartilage and bone structure were assessed by μCT, quantifying cartilage thickness, fill, and surface location, and bone-cartilage interface location. Cartilage thickness was measured as the vertical height from the cartilage surface to the calcified interface, and averaged at the three indentation sites. Cartilage fill was determined as the percent area of OP cartilage that was within the Non-OP repair site. Cartilage surface and bone-cartilage interface locations were determined as the average vertical height between Non-OP and OP cartilage and bone contours, respectively, with positive (negative) values indicating elevation (depression). Pairs of

OP and Non-OP surfaces were registered using custom MATLAB code [9], and parameters were evaluated within a 2.5mm radius of the repair center. The repair center was determined as the midpoint between visually identified discontinuities in the bone, or the corresponding Non-OP location.

Histological Processing

Osteochondral slabs, previously fixed, were decalcified, embedded in paraffin, sectioned at 7μm, deparaffinized, stained with Safranin-O or Hematoxylin/Eosin (H&E), and digitized at 20X (Aperio ScanScope, Aperio Technologies Inc., Vista, CA). The histopathology score had a 0-15 scale, with high scores corresponding to degeneration. In addition, surface irregularity and Safranin-O staining components of the histopathology score were determined separately to assess surface-specific structural degeneration [4] and relative matrix content, respectively.

Results

Cartilage Metabolism

Adequate tissue for glycosaminoglycan synthesis evaluation was only available for two FROZEN allografts, but with that limitation, biosynthesis of glycosaminoglycans by chondrocytes in cartilage varied with allograft treatment (p<0.05). ^{35}S-sulfate-incorporation in Non-OP was $23.2\pm9.3\times10^3$CPM/mg, and was similar to FRESH (p=0.8), which was $20.2\pm2.6\times10^3$CPM/mg. ^{35}S-sulfate-incorporation was ~95% lower in FROZEN than Non-OP (95% CI, 95-97%) and FRESH (95% CI, 95-96%) (p<0.01 vs. Non-OP, p<0.05 vs. FRESH), and was $0.9\pm0.1\times10^3$CPM/mg.

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CHAPTER 3:

IN VIVO EFFICACY OF FRESH VS. FROZEN

OSTEOCHONDRAL ALLOGRAFTS

IN THE GOAT AT 6 MONTHS

IS ASSOCIATED WITH PRG4 SECRETION

3.1   Abstract

The long-term efficacy of osteochondral allografts is due to the presence of viable chondrocytes within graft cartilage. Chondrocytes in osteochondral allografts, especially those at the articular surface that normally produce the lubricant proteoglycan-4 (PRG4), are susceptible to storage-associated death. The hypothesis of this study was that the loss of chondrocytes within osteochondral grafts leads to decreased PRG4 secretion, after graft storage and subsequent implant. The objectives were to determine the effect of osteochondral allograft treatment (FROZEN vs. FRESH) on secretion of functional PRG4 after (i) storage, and (ii) 6months *in vivo* in adult goats. FROZEN allograft storage reduced PRG4 secretion from cartilage by ~85% compared to FRESH allograft storage. After 6months *in vivo*, the PRG4-secreting function of osteochondral allografts was diminished with prior FROZEN storage by ~81% versus FRESH allografts and by ~84% versus non-operated control cartilage. Concomitantly, cellularity at the articular surface in FROZEN allografts was ~96% lower than FRESH allografts and non-operated cartilage. Thus, the PRG4-

secreting function of allografts appears to be maintained *in vivo* based on its state after storage. PRG4 secretion may be not only a useful marker of allograft performance, but also a biological process protecting the articular surface of grafts following cartilage repair.

3.2   Introduction

Long-term efficacy of osteochondral allografts (OCA) depends on the presence of viable chondrocytes within graft cartilage to locally maintain homeostasis and prevent degeneration. Fresh OCA have high chondrocyte viability and, when implanted into large cartilage defects, resulted in long-term clinical success rates >75% [1, 2, 4, 6, 18]. In contrast, frozen OCA are devoid of viable chondrocytes and, as used for massive oncologic joint reconstructions, result in cartilage that, in retrievals, is acellular and exhibits surface and bulk tissue degeneration [7]. While chondrocyte viability is a useful marker of overall cartilage tissue function, future improvements in the application of OCA would benefit from identification of markers of allograft biological performance and sensitive indices of cartilage function.

Since early-stage cartilage deterioration is evident at the articular surface and clinical evaluations are not sensitive to detect such changes, animal models are needed to identify biomarkers that indicate or predict cartilage degeneration in OCAs. In large animal models of OCA repair, articular surface irregularity is evident as early as 6months post-operatively with frozen grafts but not with fresh grafts [24], and progresses with increasing post-operative duration [10, 27, 31, 36], in association with cartilage softening and diminished superficial zone cellularity [23, 24]. Thus, cartilage surface health may be a critical indicator of long-term function, and examining zone-specific functions, especially in this vulnerable region, may provide additional insight into the factors necessary for graft success or failure.

Chondrocytes in the superficial zone normally secrete a lubricant molecule proteoglycan-4 (PRG4) [35], which has friction-lowering boundary lubrication function [5, 8, 12, 33]. PRG4, also found in synovial fluid [37], adsorbs to the articular surface in synovial joints, and is important for maintaining the health of the cartilage

and joint [11, 13]. Thus, death of chondrocytes in OCA may disrupt cartilage lubricant production and contribute to graft degeneration and subsequent failure. In addition, the propensity for chondrocytes within OCA to secrete functional PRG4 may be useful as a predictor of cartilage surface health and allograft biological performance.

Thus, the hypothesis of this study was that the loss of chondrocytes within OCA leads to decreased local PRG4 secretion, after graft storage and subsequent implant. To test this hypothesis, the specific aims were to determine the effect of OCA treatment (FROZEN vs. FRESH) on secretion of functional PRG4 after (i) storage, and (ii) 6months *in vivo* in adult goats.

3.3   Materials and Methods

Experimental Design

Experiment 1: OCA Storage. The effects of FROZEN and FRESH OCA storage on the (a) cumulative and (b) time-dependent rate of PRG4 secretion from adult goat cartilage were determined. After storage FROZEN at -70°C or usage FRESH within 3d, cartilage was isolated and incubated in medium supplemented with 10 ng/mL TGF-β1 to stimulate PRG4 secretion [32]. Explants were incubated for 15d to assess cumulative secretion with medium changes and collection every 2-4d, and for 7d with medium changes and collection at days 1, 3, 5, and 7 to assess time-dependent secretion. Some FRESH cartilage samples were incubated with cycloheximide (CX) to distinguish between release of existing and newly produced PRG4. Cartilage-conditioned medium (CM, n=3-4) was analyzed for PRG4 by ELISA.

Experiment 2: *In Vivo* OCA. The effects of transplantation of FROZEN and FRESH OCA after 6months *in vivo* on subsequent PRG4-secreting function and surface cellularity were determined using tissue from a previous study [24]. Portions of cartilage from FROZEN and FRESH OCA at medial femoral condyle (MFC) and lateral trochlea (LT) sites, along with site-matched regions of non-operated (Non-Op) joints and cartilage at the lateral femoral condyle (LFC) site, were retrieved after 6months *in vivo* (n=3-7 at each site) and analyzed for PRG4 secretion and cellularity. CM was analyzed for PRG4 by ELISA and Western blot, as well as functionality of the produced lubricant with a cartilage-on-cartilage friction test.

OCA Storage

OCA were prepared from adult Boer goats (2-4yo) and stored FROZEN or FRESH. Some osteochondral samples were stored FROZEN at -70°C for 10days. Other fragments were stored FRESH at 4°C for 3days in tissue culture medium (low-glucose Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 0.1mM non-essential amino acids, 2mM L-glutamine, 25μg/mL L-ascorbic acid, and antibiotics-antimycotics (100U/mL penicillin, 100μg/mL streptomycin, and 0.25μg/mL fungizone, PSF). For cumulative PRG4 secretion in Exp 1, FROZEN and FRESH donor OCA were stored as LFC fragments (each, n=4 joints) from both knees of adult Boer goats (n=4 animals) described previously [24]. Following storage, explants (d=5mm) were used for subsequent analysis. For time-dependent PRG4 secretion in Exp 1, FROZEN and FRESH osteochondral cores (d=3.2mm, n=27 cores) were prepared from both shoulders (n=2 joints) of an adult Boer goat (3yo) [22], and 3 cores/sample (n=3 samples/group) were pooled for analysis.

Cartilage Culture and Conditioned Medium (CM)

Cartilage from OCA was removed from the bone and incubated in media containing TGF-β1 to stimulate chondrocyte PRG4 secretion [32], and collected for subsequent analysis. Following storage or retrieval, cartilage explants, with the articular surface intact, were incubated in medium (low-glucose Dulbecco's modified Eagle's medium, 10mM HEPES buffer, 0.1mM non-essential amino acids, 0.4mM L-proline, 2mM L-glutamine, 25μg/mL L-ascorbic acid, and PSF) containing 10ng/mL TGF-β1 and 0.01% bovine serum albumin for 7-20days at 37°C in an atmosphere with 5% CO_2. For cumulative PRG4 secretion in Exp 1, CM was pooled over 15days. For time-dependent PRG4 secretion in Exp 1, CM was collected at days 1, 3, 5, and 7, and additional FRESH samples (n=3 cores) were also incubated subsequently in medium

but with additional cycloheximide 100μg/mL CX to inhibit biosynthesis [30]. For *in vivo* PRG4-secreting function of OCA in Exp 2, CM was pooled over 20days. Medium (surface area/volume/day=$0.75cm^2$/mL/d) was replaced every 2-4days, and CM was collected for subsequent analysis.

Quantification and Characterization of PRG4 in CM

The CM collected from cartilage cultures was analyzed for PRG4 by indirect ELISA and/or Western blot, as previously described [14]. Briefly, for ELISA, portions of CM was diluted serially, adsorbed, and then reacted with monoclonal antibody 3-A-4 (MD Bioproducts, St. Paul, MN), horseradish peroxidase-conjugated secondary antibody, and ABTS substrate. Rates of PRG4 secretion were calculated by normalizing the total mass of PRG4 in the CM to cartilage surface area and incubation duration. Briefly, for Western blot, portions of CM were pooled, separated by horizontal agarose gel electrophoresis, transferred to polyvinyidene difluoride membrane, and probed with 3-A-4, with ECL-Plus detection. Luminescence from the membrane was digitized with STORM 840 Imaging System (Molecular Dynamics, Fairfield, CT).

Friction-Lowering Lubricant Function of CM

To determine functionality of the produced lubricant, CM was analyzed for friction-lowering function, as indicated by kinetic coefficient of friction (μ) in a cartilage-on-cartilage friction test, as previously described [34]. To prepare CM for friction tests, portions were dialyzed (6-8,000 MWCO) exhaustively against distilled water, lyophilized, and resuspended in PBS with proteinase inhibitors (PI, 2 mM di-sodium ethylenediamine tetraacetate, 5 mM benzamidine-HCl, 10mM N-

ethylmaleimide, and 1 mM phenylmethanesulfonyl fluoride) to concentrate samples by 2x. Friction tests were conducted with PBS as a negative control, the CM samples, 3% normal caprine synovial fluid (SF) with PI (the dilution to approximately match the [PRG4] in Non-Op CM), and full-strength bovine SF (100%SF) as a positive control. From torque and axial load, μ was calculated [34].

Retrieved Cartilage Cellularity

Cellularity was assessed at the articular surface of retrieved OCA by *en face* imaging after nuclear staining, as described previously [22]. Briefly, cell nuclei were labeled with PicoGreen (0.2 μg/mL in PBS), and the articular surface was imaged with a 10X objective over a 1.5 x 1.5 mm^2 area by confocal microscopy to a depth of 30μm. Image stacks were processed with a custom routine to segment and quantify the number of cells. Cellularity (cells/cm^3) was calculated as the number of cells divided by cartilage volume.

Statistics

Data are presented as mean±SEM, with $p < 0.05$ considered significant. In Exp 1, the effect of OCA storage (FROZEN vs. FRESH) on cumulative PRG4 secretion was assessed by 2-tailed *t*-test, and the effect of OCA storage (FROZEN, FRESH, FRESH+CX) on time-dependent PRG4 secretion was assessed by two-way ANOVA, with time (days 1, 3, 5, and 7) as a repeated factor. In Exp 2, the effects of OCA storage (Non-Op, FROZEN, FRESH) and site (MFC, LT) on PRG4 secretion and cellularity were assessed by ANOVA, followed by Tukey *post hoc* tests to determine which experimental group means differed from each other. Also in Exp 2, for Non-Op, the effect of site (MFC, LT, LFC) on PRG4 secretion was determined by one-way

ANOVA, and at LFC, the effect of knee (Op vs. Non-Op) on PRG4 secretion was determined by t-test. The effect of lubricant on μ was assessed by t-test, with Bonferroni adjustment to reflect multiple (5) comparisons. At each site, OCA storage groups (Non-Op, FROZEN, FRESH) were compared to each other and to positive (SF) and negative (PBS) controls. Statistical analyses were performed using Systat 10.2 (Systat Software, Chicago, IL).

3.4   Results

Experiment 1: OCA Storage

FROZEN OCA storage reduced PRG4 secretion by cartilage explants isolated from such samples, compared to FRESH OCA storage. After FROZEN OCA storage, cumulative PRG4 production over 15d was reduced by ~85% versus that from FRESH stored samples (1.95 $\mu g/(cm^2 \cdot d)$, p<0.05, Fig. 3.1A).

During the initial 7d of incubation, PRG4 secretion varied with OCA storage and cartilage incubation conditions (p<0.001) and with incubation time (p<0.001), with significant interaction (p<0.001). PRG4 release from FRESH cartilage ranged from 1.84−4.55 $\mu g/(cm^2 \cdot d)$ at days 1, 3, 5, and 7, and was substantially higher than that from FRESH+CX and FROZEN cartilage (each, p<0.001, Fig. 3.1B). PRG4 release from FROZEN cartilage was similar to that of FRESH+CX (p=0.3−1.0), with most of the PRG4 release from these samples occurring by day 1 (0.22 $\mu g/(cm^2 \cdot d)$, p<0.01 vs. days 3, 5, and 7). Thus, of the PRG4 released from FRESH cartilage, most was accounted for by newly synthesized protein, above that released from cartilage previously FROZEN or treated with CX to block protein synthesis.

Experiment 2: In Vivo OCA

After 6months *in vivo*, PRG4 secretion from cartilage of the retrieved OCA varied with allograft storage (p<0.001), but not with site on the knee (p=0.2). At MFC and LT, PRG4 secretion in Non-Op was similar to FRESH (each, p>0.4, Fig. 3.2A). PRG4 secretion for Non-Op were similar between sites (each, p>0.8), and was 2.60, 2.85, and 2.71 $\mu g/(cm^2 \cdot d)$ at MFC, LT, and LFC, respectively. In addition, at LFC, PRG4 secretion was similar between Non-Op and Op joints (p>0.7, Fig. 3.2A). In contrast, PRG4 secretion after FROZEN storage was only 0.20 $\mu g/(cm^2 \cdot d)$ at MFC

and 0.67 µg/(cm^2·d) at LT, and was markedly reduced versus Non-Op, by ~92% at MFC (p<0.01) and by~76% at LT (p<0.05), with trends of a reduction compared to FRESH, by ~88% at MFC (p=0.1) and by ~74% at LT (p=0.1).

Qualitatively, the CM contained a component consistent with PRG4, as evidenced by a major immunoreactive band on Western blot with monoclonal antibody 3-A-4. This immunoreactive band was evident for CM from Non-Op and FRESH OCA, and lighter for FROZEN OCA. The localization of immunoreactive material on Western Blot and the difference in band intensities between samples from different groups (Fig. 3.2B) appeared consistent with the differences in PRG4 secretion determined by ELISA (Fig. 3.2A).

Concomitantly, chondrocyte cellularity varied with OCA treatment (p<0.001), but not with site (p=0.3). Cellularity at the surface of Non-Op samples was similar to those of FRESH samples (p>0.7), and was 148x10^{6}cells/cm^3 at MFC and 145x10^{6}cells/cm^3 at LT. Cellularity in FROZEN was ~96% lower than Non-Op and FRESH OCA, and was 6x10^{6}cells/cm^3 at MFC (each p<0.001, Fig. 3.3A) and 5x10^{6}cells/cm^3 at LT (each p<0.001, Fig. 3.3B).

Lubricating function of CM from cartilage of retrieved OCA after 6months *in vivo* was evident for OCA that had been stored FRESH, but diminished by prior FROZEN storage. For cartilage from MFC and LT sites, µ of the CM from Non-Op was similar to FRESH (each, p>0.5), and 3%SF (each, p>0.4), and markedly better (lower) than that of PBS (each, p<0.001, Fig. 3.4). In contrast, in cartilage from both MFC and LT sites, µ of the CM from FROZEN retrievals was markedly worse (higher) than that of Non-Op (each, p<0.05), and that of 3%SF (each, p<0.01), and not different from PBS (each, p>0.2, Fig. 3.4). Thus, the lubricating function of molecules secreted by cartilage from OCA was dependent on the FRESH vs FROZEN storage

conditions, paralleling their propensity to secrete PRG4, as described above.

3.5   Discussion

These results demonstrate that the OCA storage conditions that affect chondrocyte viability also affect the subsequent secretion of functional PRG4 secretion, not only immediately after storage and but also after implantation and retrieval 6months post-operatively in goat knees. After 6 months *in vivo*, the cartilage of adult goat osteochondral samples that were stored FRESH contained a high density of chondrocytes at the articular surface (Fig. 3.3), secreted PRG4 into culture medium at a high rate both after storage (Fig. 3.1) and after retrieval (Fig. 3.2), and imparted lubrication function to the culture medium (Fig. 3.4). In contrast, the cartilage of FROZEN OCA was devoid of surface chondrocytes (Fig. 3.3), released PRG4 at a low rate after retrieval *in vivo* (Fig. 3.2) consistent with that of FROZEN-stored cartilage prior to implantation (Fig. 3.1), generating culture medium with lubricating (dys)function similar to PBS (Fig. 3.4). Thus, the PRG4-secreting function of the cartilage of OCA is maintained *in vivo* in a manner that reflects its state after *in vitro* storage before implantation.

The use of the adult goat model to study markers and mediators of cartilage surface health associated with OCA storage involved the consideration of a number of issues. To probe the PRG4 secretory responses, the *in vitro* culture conditions were chosen to vigorously stimulate PRG4 secretion [32] and to produce sufficient media volume for analysis. The PRG4 secretion rates during *in vitro* culture may differ from those *in vivo*. In addition, the retrieved samples were one frozen and one fresh OCA from each individual knee. This provided a common joint environment for samples from both groups so that the properties of the retrieved OCA reflected activities as regulated by local properties and the overall joint environment.

The high rates of PRG4 secretion and lubricating ability of CM, in conjunction

with high surface cellularity, for FRESH but not FROZEN OCA suggests that PRG4-secreting function may be a useful marker of biological function following cartilage repair. These FRESH OCA, which maintained depth-dependent tissue homeostasis at 6months, had similar mechanical, cellular, and matrix properties to Non-Op site-matched cartilage, and preserved a smooth articular surface [24]. While biomarkers, characteristics that objectively measure and evaluate normal biological processes, have been studied extensively for osteoarthritis [15], biomarkers for pre-clinical models of cartilage repair have not. PRG4 biosynthesis would appear to be a useful biomarker, since in cartilage, it depends both on the presence of chondrocytes normally present at the articular surface and also exhibits mechanosensitivity [9, 19-21, 41]. Repair strategies that target restoration of the normal biology of the cartilage surface function may be advantageous since PRG4 secretion contributes to maintenance of normal joint function and cartilage homeostasis. Thus, the PRG4-secreting function of cartilage following repair may serve as an important index of long-term function and clinical efficacy in OCA and following other cartilage repair therapies.

Reduced PRG4 secretion by FROZEN OCA, in association with increased irregularity of the articular surface at 6 months [24], suggests that local PRG4 secretion may be one mechanism by which the articular surface of grafts is protected following cartilage repair. Such FROZEN OCA exhibited deterioration at 6 months with cartilage softening, loss of chondrocytes, reduced proteoglycan content, and visible structural deterioration, likely indicating progression toward failure [24]. Cartilage of FROZEN OCA may experience increased mechanical loads as a result of local boundary lubricating (dys)function that leads to accelerated deterioration of the articular surface. PRG4 protects the cartilage surface from wear by reducing friction

[12] and preserves superficial zone chondrocyte viability in mouse models of PRG4-deficient joints [3, 28]. Thus, implanting OCA that produce sufficient PRG4 may be critical to maintain a low-friction articulating surface, prevent cartilage deterioration, and improve overall OCA performance. On the other hand, it is possible that PRG4 within the SF is the critical factor, and the relatively small decrease in area of PRG4-secreting chondrocytes was not the major cause of local deterioration. Indeed, the SF within the knee appeared to contain PRG4 sufficient to coat the joint surface, since FROZEN OCA released some PRG4 during *in vitro* culture.

The association of PRG4 secretion in cartilage of OCA with its state after storage suggests that PRG4 secretion could be useful for screening OCA prior to implantation. Although PRG4 has been localized to the articular surface of repair tissue after autologous chondrocyte implantation, the surface staining was likely from the PRG4 in the joint synovial fluid, as there was minimal cellular PRG4 expression [29]. During routine 4°C storage of OCA, chondrocytes, especially those at the articular surface with PRG4-secreting function, are susceptible, with increasing storage duration, to cell death [25, 26, 38, 39]. While usage of 4°C stored OCA leads to some clinical improvement compared to pre-operative patient function, the long-term efficacy of 4°C stored OCA in animal models suggests that such grafts are not as effective as fresh grafts, and susceptible to deterioration with variable outcomes [16, 17, 40], supporting the importance of maintaining cell viability during OCA storage. Screening stored OCA for PRG4-secretion prior to implantation and/or storing OCA in a manner that preserves this function of cartilage may be useful to improve the long-term biological performance of osteochondral allografts.

3.6   Acknowledgments

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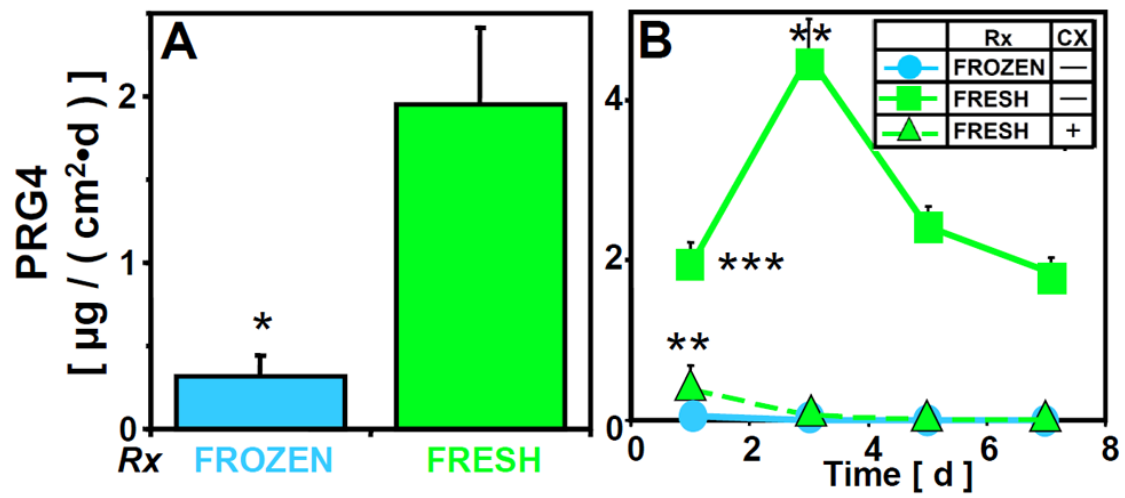
Figure 3.1: Effect of allograft storage on rate of PRG4 secretion from OCA. (**A**) Cumulative release, n=4 animals, *p<0.05. (**B**) Time-dependent release and effect of cyclohexamide (CX), n=3/condition, **p<0.01, ***p<0.001.
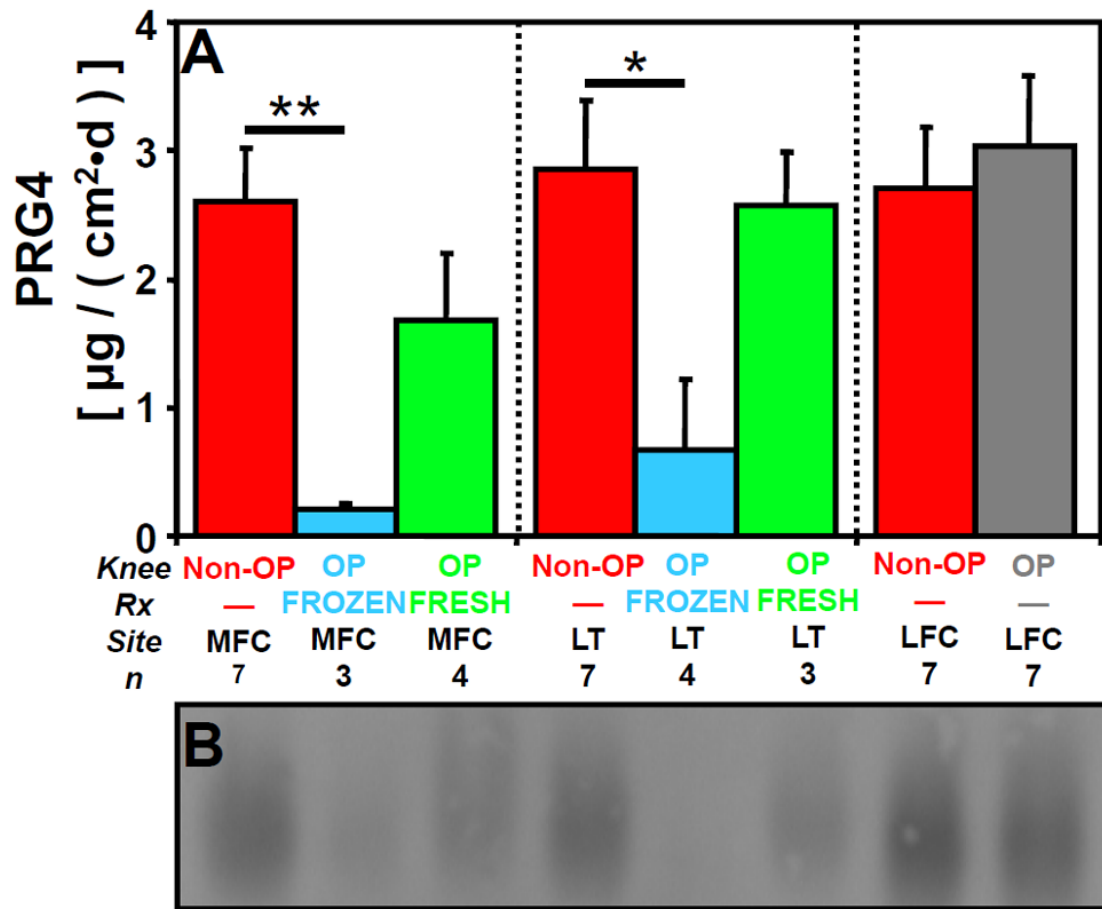
Figure 3.2: Effect of transplantation of OCA after 6months *in vivo* on subsequent rate of PRG4 secretion in culture. (**A**) Quantification by ELISA, n=3-7, *p<0.05, **p<0.01. (**B**) Characterization by Western blot.
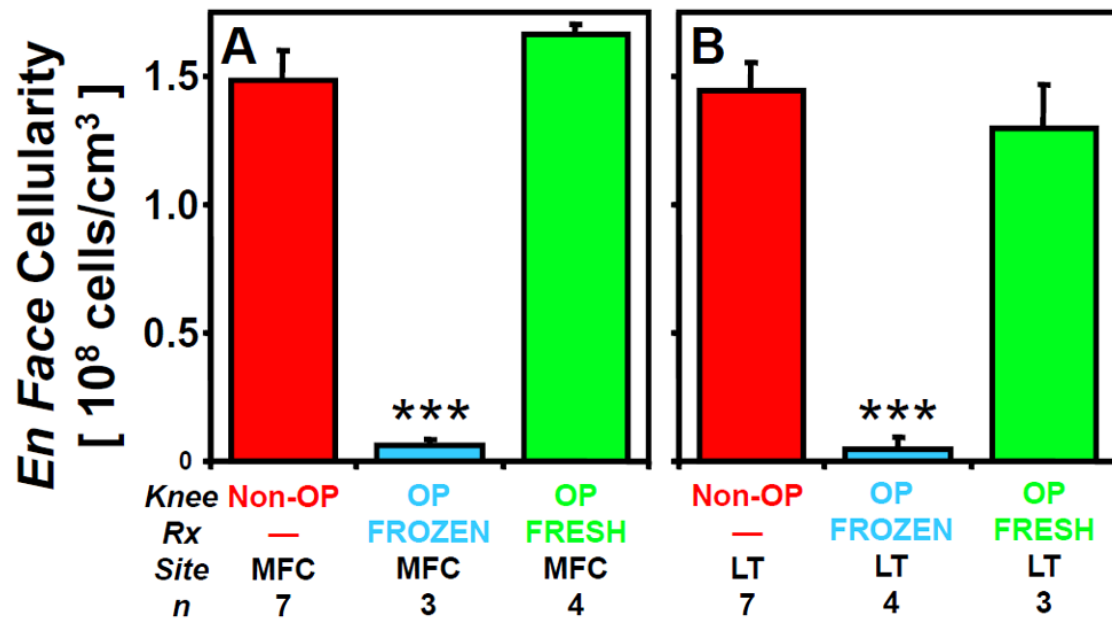
Figure 3.3: Effect of storage and transplantation of OCA after 6months *in vivo* on cellularity at the cartilage surface *en face*. n=3-7, ***p<0.001

Knee	—	Non-OP	OP	OP	Non-OP	OP	OP	—	—
Rx	—	—	FROZEN	FRESH	—	FROZEN	FRESH	—	—
Site	—	MFC	MFC	MFC	LT	LT	LT	—	—
LUB	PBS	CM	CM	CM	CM	CM	CM	3%SF	100%SF
n	14	7	3	4	7	4	3	14	14
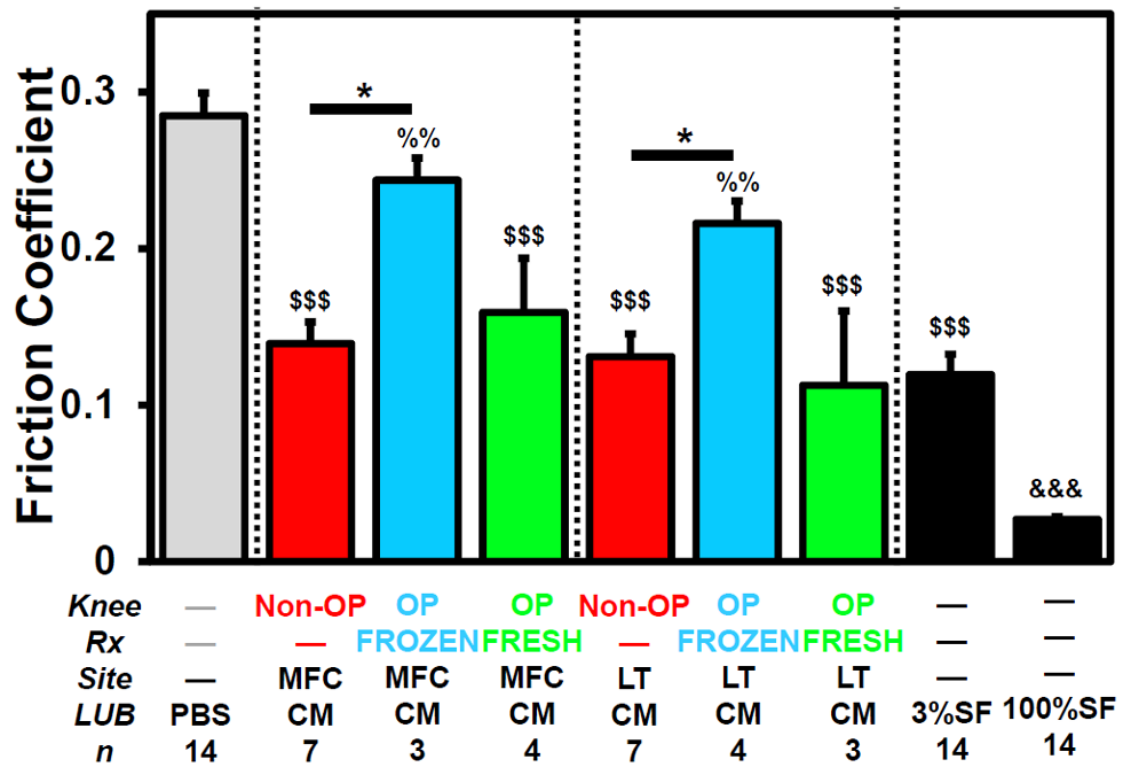
Figure 3.4: Effect of transplantation of OCA after 6months *in vivo* on lubricant property of cartilage conditioned medium (CM). Friction coefficient determined in cartilage-on-cartilage friction test of lubricant solutions: PBS, CM, 3% caprine synovial fluid (3%SF), 100% bovine synovial fluid (100%SF). n=3-14, *p<0.05, $$$p<0.001 vs. PBS, %%p<0.01 vs. 3%SF, &&&p<0.001 vs. all others.

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CHAPTER 4:

THE IN VIVO PERFORMANCE
OF OSTEOCHONDRAL ALLOGRAFTS IN THE GOAT
IS DIMINISHED WITH EXTENDED STORAGE AND
DECREASED CARTILAGE CELLULARITY

4.1   Abstract

Background: Currently, osteochondral allografts (OCA) are typically used after 4°C-storage for prolonged durations (15-43days), which compromises chondrocyte viability, especially at the articular surface. The long-term *in vivo* performance of these fresh-stored allografts, in association with variable cellularity, is unknown.

Hypothesis/Purpose: Determine the effect of 4°C-storage duration (14, 28days) versus the best (fresh) and worst (frozen) conditions of chondrocyte viability on structure, composition, and function of cartilage in the goat, and the association of retrieved chondrocyte cellularity with those tissue properties.

Study Design: Controlled Laboratory Study

Methods: The effect of allograft storage on *in vivo* repair outcomes was determined for OCA transplanted into fifteen recipient goats and analyzed at 12months. Repair outcomes were assessed by examining cartilage structure (gross, histopathology), composition (cellularity by depth, matrix fixed charge), and

biomechanical function (stiffness). Relationships between cellularity and structural scores, matrix fixed charge, and stiffness were assessed by linear regression.

Results: Repair outcomes in 4°C-stored OCA were similar after 14d and 28d storage, and both were inferior to fresh OCA, and were accompanied by diminished cellularity at the surface, matrix fixed charge, and histopathological structure. Overall, cellularity by depth and matrix fixed charge in cartilage of fresh OCA were similar to non-operated controls. However, cellularity at the articular surface and matrix fixed charge in 4°C-stored OCA were lower than fresh, by ~55% (95%CI, 32-76%) and ~20% (95%CI, 9-30%), respectively. In frozen OCA, cellularity and matrix fixed charge were lower than 4°C-stored OCA, by ~93% (95%CI, 88-99%) and ~22% (95%CI: 10-35%), respectively. Cellularity correlated negatively with cartilage health indices, including structural scores, and positively with matrix fixed charge and stiffness.

Conclusion: Reduced cellularity at the articular surface, resulting from 4°C storage, was associated with variable long-term outcomes, versus consistently good repair by fresh allografts. Cellularity at the articular surface was an important index of biological performance.

Clinical Relevance: Normal chondrocyte density *in vivo,* especially in the superficial region of cartilage, is important for maintaining long-term cartilage function and matrix content. In human cartilage, containing cells at ~3-5x lower density than goat, repair outcomes may be related to absolute minimum number of cells rather than density.

4.2   Introduction

Osteochondral allografting is a desirable treatment option to repair large articular defects, providing functional restoration of the affected joint. Traditionally, osteochondral allografts were implanted fresh, within 7days, and have high chondrocyte viability at implantation. Usage of such fresh allografts has resulted in long-term clinical success rates exceeding 75% [4, 7, 9, 12, 24]. Currently in the United States, osteochondral allografts are stored at 4°C to accommodate commercial screening and processing protocols, with testing typically requiring at least 14days [1, 20, 44]. Such fresh-stored osteochondral allografts are routinely used after 4°C storage for prolonged durations (15-43days), which compromises chondrocyte viability, and has resulted in some short-term clinical improvement compared to pre-operative function for small cohorts of patients [11, 20, 23, 44]. However, long-term *in vivo* performance of these fresh-stored allografts, in association with variable cellularity, is unknown.

The long-term efficacy of osteochondral allografts is thought to be due to the presence of viable chondrocytes within graft cartilage which preserves tissue homeostasis and prevents degeneration. Preserving chondrocyte viability during allograft insertion, as well as storage, may be critical to graft function. Significant impaction forces can sometimes be generated during insertion of osteochondral allografts, and such supra-physiologic loading and other traumatic injury has deleterious effects on chondrocyte viability, especially at the articular surface [6, 15, 19, 31, 39, 41]. In addition, during prolonged 4°C storage, chondrocytes, particularly in the superficial zone of cartilage, succumb with increasing storage duration; chondrocyte viability deteriorates substantially in the superficial zone by 14days [3, 27], and overall by 28days [3, 27, 32, 33, 42, 43]. While shorter 4°C storage durations

(i.e. 14days) preserve overall chondrocyte viability, acquisition and distribution of these allografts is logistically challenging. Significant loss of viable chondrocytes at longer 4°C storage durations (i.e. 28days) may contribute to graft degeneration and subsequent failure, but the increased storage duration allows for easier distribution of allografts. Investigating the relationship between these critical storage durations and repair outcomes may expand/limit the definition of suitable graft tissue.

Determinants of long-term allograft function can be pursued using multi-scale analyses of cartilage repair in animal models. In such animal models, allografts stored at 4°C for 14days appear grossly, biochemically, and biomechanically similar to fresh allografts at short post-operative durations up to 3months [26], but exhibit mild to moderate gross degenerative changes at 6 and 12months [34, 37]. Allografts stored at 4°C for >21days have variable outcomes; some grafts tend to undergo severe degeneration, while others tend to be similar in appearance to fresh grafts and grafts stored at 4°C for 14days [21, 34]. At 12months, some 4°C stored allografts appear qualitatively to have diminished cellularity at the cartilage surface [34], which may be an important predictor of long-term graft efficacy. While storage duration did not correlate with histologic appearance at 12months [34], other determinants of repair outcomes have not been investigated. Quantitative analysis of cellularity in retrievals and its relationship to graft efficacy may expand the current understanding of *in vivo* maintenance of cartilage structure, composition, and function.

Thus, the hypothesis of this study was that decreased chondrocyte viability in stored osteochondral allografts is associated with poor repair outcomes. The specific aims were to determine the effect of allograft storage on the macroscopic structure, cellular and matrix fixed charge composition, and biomechanical function of cartilage within osteochondral allografts retrieved at 12months in a goat model, and to assess

the associations between chondrocyte cellularity and cartilage structure, matrix fixed charge, and biomechanical stiffness.

4.3   Materials and Methods

Experimental Design

Adult Boer goats (n=16, 2-3yo) were operated (OP) in one knee with Institutional Animal Care and Use Committee approval. Each OP knee received two site-matched osteochondral allografts, one implanted into the medial femoral condyle (MFC) and another one into the lateral trochlea (LT) sites; grafts of different storage treatments (FROZEN, FRESH, or stored at 4°C for 14days or 28days, Fig. 4.1) were implanted alternatingly into MFC and LT. Contralateral knees were Non-Operated (Non-OP) controls. At 12months, animals were euthanized and both knees analyzed, assessing macroscopic and histologic structure, cellular and matrix composition, and biomechanical function.

Donor Preparations

Donor osteochondral allografts were prepared from both knees of adult Boer goats (n=8, 2-4yo). Hind limbs were received on wet ice within 24hours of sacrifice. Under aseptic conditions, each knee was harvested and divided into condyle and trochlea fragments. Fragments were thoroughly rinsed with phosphate-buffered saline (PBS [Mediatech, Inc., Manassas, VA]) supplemented with antibiotics-antimycotics (100U/mL penicillin, 100μg/mL streptomycin, and 0.25μg/mL fungizone, PSF [Life Technologies, Grand Island, NY]). Some fragments (n=8) were stored FROZEN at -70°C for 10days. Other fragments (each, n=8) were stored at 4°C in tissue culture medium (low-glucose Dulbecco's modified Eagle's medium [GIBCO, North Andover, MA], 10% fetal bovine serum [Omega Scientific, Tarzana, CA], 0.1mM non-essential amino acids [Sigma-Aldrich, St. Louis, MO], 2mM L-glutamine [Life Technologies,

Grand Island, NY], 25μg/mL L-ascorbic acid [Sigma-Aldrich, St. Louis, MO], and PSF) for 3days (FRESH), 14days (4°C/14d), or 28days (4°C/28d).

Allograft Surgery

Osteochondral allografting was performed through a medial knee arthrotomy under general anesthesia, as previously described [28]. Briefly, the knee joint was exposed through a medial parapatellar incision, and lateral patella dislocation. An osteochondral defect (d=7.5mm, h=5mm) was created at MFC and LT sites under continuous saline irrigation. An orthotopic cylindrical osteochondral plug (d=8mm, h=5mm) was harvested from donor tissue, thoroughly cleansed with saline, and inserted carefully into the defect with gentle impaction until the graft was flush with the surrounding articular surface, using a graft tamp and load-cell instrumented mallet. Following closure, OP knees were cast in a modified Schroeder-Thomas splint for 13days to limit weight-bearing [25].

Animals were monitored post-operatively for pain and lameness throughout the study. After 12months, animals were euthanized with potassium chloride (1-2mmol/kg) intravenously under stage III anesthesia. One animal was sacrificed at 2months due to a lateral luxated patella, and analysis was not included in the study.

Surgical Graft Insertion

Graft insertion parameters, including impaction force (peak and average), impulse (average and total), tap duration, and number of taps, were measured using a mallet instrumented with a dynamic force sensor (208C05, PCB Electronics, Depew, NY) to allow loads to be recorded during gentle mallet impaction [6]. Force-time data was acquired at 50 kHz, and processed to determine impaction parameters. The

loading force (N) was taken as the peak or average of all taps, the magnitude of the load impulse (N·s) was calculated as the area under the force-time curve for each tap and all taps, and the impact duration (ms) was calculated as the average interval between half-maximum loads.

Repair Site Analysis

Intact knee joints were received on wet ice within 24hours of sacrifice. Distal femurs were harvested, photographed, and examined grossly. Next, osteochondral cores (d=15mm, h~8-10mm) were isolated to encompass the experimental site, using a custom coring bit under irrigation with cold PBS+PSF. Samples were tested biomechanically, and then osteochondral slabs (width=2mm), cut centrally from proximal to distal, were isolated. Osteochondral slabs were fixed in 4% paraformaldehyde, and then analyzed for cellularity, matrix fixed charge, and histopathology index.

Macroscopic Structure

Macroscopic structure of repair tissue was evaluated grossly for surface texture, defect filling/size, and graft-recipient cartilage integration. The gross-score had a 0-9 scale, with high scores corresponding to degeneration and/or poor repair [13]. Scores were averaged from two blinded observers. Surface texture was scored for the central region of the repair tissue, from normal (score=0) to <50% normal (score=3). Defect filling/size was scored based on surface congruity and repair size, as normal/flush with d~8mm (score=0), flush with 5mm≤d<8mm (score=1), flush with d<5mm or depressed with 5mm≤d<8mm (score=2), and depressed with d>5mm (score=3). Graft-recipient cartilage integration was scored based on transition across

the host and repair region, from complete integration (score=0) to <50% smooth transition (score=3).

Cartilage Composition

Cellularity was assessed with depth from the articular surface. Cell nuclei were labeled fluorescently, and full-thickness cartilage was imaged along a vertical profile to a depth of 45μm at 1.5μm intervals by confocal microscopy (Leica TCS SP5, Buffalo Grove, IL). Image stacks were processed in three-dimensions with a custom routine to localize and count cells [17]. The articular surface was segmented manually from a single background image, which clearly delineated the boundaries of cartilage. Cellularity was calculated as the number of cells divided by cartilage volume (cells/cm^3) from the segmented articular surface (by 50 or 100μm intervals). Cellularity at selected intervals (0-50, 51-200, 201-600μm) from the articular surface was reported. Cellularity at all 50-100μm bins at depths up to 600μm are reported in the Appendix. Cellularity at depths >600μm, in thicker cartilage samples, was similar to cellularity at depth=500-600μm (data not shown). Representative cellularity images were illustrated by merging five fluorescent images from the stack (at 0, 7.5, 15, 22.5, and 40.5μm) to form one single image. For correlations, cellularity was calculated for superficial, middle, and deep zones of cartilage, defined as 0-15%, 15-50%, and 50-100% of the cartilage thickness.

Matrix fixed charge in cartilage was assessed by Hexabrix-Enhanced micro-computed tomography (HE-μCT), as previously described.[28] Hexabrix, an ionic contrast agent, distributes inversely to the fixed charge density in soft tissues, and is therefore a sensitive (inverse) indicator of proteoglycan content [30]. Briefly, fixed osteochondral slabs were incubated for ~1day in a Hexabrix (20%, by volume in PBS)

solution, and then imaged in 3D using μCT (Skyscan 1076, Kontich, Belgium): 70kV, voxel=$(18\mu m)^3$. HE-μCT value, expressed as percent, was determined by comparing the imaged x-ray attenuation value to Hexabrix phantoms, fit by linear regression, within the segmented cartilage region (width=2mm). The spatial variation in matrix fixed charge was illustrated with color maps representing 0-20% HE-μCT value within the cartilage.

Cartilage histopathology was assessed with the modified Mankin score [38] using Safranin-O and Hematoxylin/Eosin (H&E) stained slides because osteochondral allograft repair resembles normal hyaline cartilage structure at the time of implant. Previously fixed osteochondral slabs were decalcified, embedded in paraffin, sectioned at 7μm, deparaffinized, stained, and digitized at 20X (Leica SCN400, Buffalo Grove, IL). The histopathology-score had a 0-15 scale, with high scores corresponding to degeneration. Scores were averaged from two blinded observers.

Biomechanical Function

Cartilage load-bearing function was assessed by indentation testing at the center of each osteochondral core. Using a benchtop mechanical tester (v500cs, Biomomentum Inc., Canada), samples were compressed rapidly by 100μm, at three sites 0.5mm apart (proximal to distal) [22]. The peak load was divided by the indentation depth, normalized to cartilage thickness and indenter tip area, and three points averaged to determine the indentation material stiffness, expressed in units of MPa.

Statistical Analysis

Data are presented as mean±SEM. For each Non-OP knee (n=15), metrics at MFC and LT (except cartilage stiffness and thickness) were averaged. The effect of allograft storage (Non-OP, FROZEN, FRESH, 4°C/14d, 4°C/28d) on cartilage cellularity and HE-μCT value, were determined by ANOVA. For cellularity, depth (0-50μm, 51-200 μm, 201-600μm) from the articular surface was considered a repeated measure, and a 2-way repeated measures ANOVA was performed. For metrics (i.e. cartilage stiffness and thickness) that varied at MFC and LT in Non-OP, effect of allograft storage and site (MFC, LT) were determined by 2-way ANOVA. Tukey post-hoc comparisons were performed to compare treatments with significant differences (p<0.05). For nonparametric data (i.e. gross- and histopathology-scores), samples were analyzed analogously using Kruskal-Wallis and Dunn's tests. Power analysis (α=0.05 and 1-β=80%) was performed with superficial chondrocyte cellularity as the primary endpoint, based on variability (SD=670cells/mm^2) from previous studies [27] and an effect size of 1.6, which was sufficient to distinguish superficial chondrocyte viability in 4°C/28d stored osteochondral allografts from fresh controls in previous in vitro studies [27]; the resulting sample size was estimated to be n=8 per treatment group. For secondary endpoints where effect size may be smaller or which differed at sites, power may not be sufficient to detect significant differences.

The relationships between indices of cartilage health and chondrocyte cellularity were assessed by regression. Gross- and histopathology-score were correlated with cellularity by nonparametric Spearman's rank method [14]. HE-μCT value (inverse of matrix fixed charge) and cartilage stiffness were correlated with cellularity by linear regression. Coefficients of determination, R^2 (parametric) and ρ^2 (nonparametric), are reported for significant relationships (p<0.05).

4.4   Results

Surgical Graft Insertion

Graft insertion parameters (mean±SD), including impaction force, impulse, tap duration, and number of taps, did not vary with allograft storage (p>0.4). Allografts were inserted with 36±20 taps, for a total impulse of 6.77±3.35N·s. For graft insertions, each tap was moderate, with peak and average forces of 415±14N and 197±7N, respectively, and impulse of 0.20±0.03N·s for 2.71±3.93ms duration. Two grafts required 96 and 112 taps, which is about three standard deviations away from the mean number of taps.

Cartilage Composition - Cellularity

Quantitatively, chondrocyte cellularity varied with allograft storage (p<0.001), with depth from the articular surface (p<0.001), and with a significant interaction effect (p<0.001). Cellularity in Non-OP was similar to FRESH (p>0.9) decreasing throughout the cartilage depth (see §4.7 Fig. 4.S1), and was $23.7 \pm 1.7 \times 10^6$cells/cm^3 for depth=0-50µm, $23.0 \pm 1.8 \times 10^6$cells/cm^3 for depth=51-200µm, and $13.3 \pm 0.9 \times 10^6$cells/cm^3 for depth=201-600µm from the articular surface (Fig. 4.2). At the articular surface for depth=0-50µm, cellularity in 4°C/14d and 4°C/28d stored allografts was ~60% lower than Non-OP (95% Confidence Interval [CI]: 49-72%; each, p<0.001), and tended to be ~55% lower than FRESH (95%CI: 32-76%; each, p=0.07). At depth=51-200µm and 201-600µm, cellularity in 4°C/14d and 4°C/28d stored allografts was similar to Non-OP and FRESH (each, p>0.7), but ~98% higher than FROZEN (95%CI: 95-100%; p<0.001 vs. all storage treatments).

Representative confocal images and histological sections of cartilage within the graft region agreed qualitatively with the above quantitative cellularity data. Non-

OP and FRESH allografts had typical chondrocyte organization, with flattened cells at high density near the articular surface and columns of cells at lower density in the deeper regions of cartilage (Fig. 4.3A,C,F,H,K,M,P,R). In 4°C/14d and 4ºC/28d stored allografts, cellularity was diminished at the articular surface, but similar to Non-OP and FRESH in deeper regions of cartilage; confocal images lacked flattened cells (Fig. 4.3D-E,N-O), and histological sections showed empty cellular lacunae at the articular surface (Fig. 4.3N1-O1,S1-T1). FROZEN allografts were acellular in both confocal images and histological sections (Fig. 4.3B,G,L,Q).

Macroscopic Structure

Gross-score varied with allograft storage (p<0.001). Overall joints appeared macroscopically normal, with no osteophyte formation or extensive degeneration (Fig. 4.4A-D). Non-OP gross-score was 0.3±0.1, and was lower than all other allograft storage treatments (each, p<0.001, Table 1). Gross-score in FRESH was consistently low at MFC (range 2-3, Fig. 4.4F), but was more variable at LT (range 3-8, Fig. 4.4J) and similar to 4°C/14d (range 2-7) and 4°C/28d (range 3-9) stored allografts (Fig. 4.4G-L, p>0.9). Gross-score was 7.4±0.7 in FROZEN (Fig. 4.4E-I), and higher than FRESH, 4°C/14d, and 4°C/28d (p<0.01 vs. FRESH, p<0.001 vs. 4°C/14d, p<0.05 vs. 4°C/28d, Table 4.1). Surface texture was similar among FRESH, 4°C/14d, and 4°C/28d (p>0.6). Defect filling/size tended to be worse in 4°C/28d versus 4°C/14d stored allografts (p=0.1), but still better than in FROZEN (p<0.05). All implanted allografts remained clearly demarcated from the surrounding host cartilage at retrieval; 4°C/14d and 4°C/28d stored allografts had worse cartilage integration scores than FRESH at MFC (each, p<0.01, §4.7 Table 4.S1).

Histopathological Structure

Histopathology-score varied with allograft storage (p<0.001). Non-OP histopathology score was 3.4±0.4, similar to FRESH (p>0.9), and lower than FROZEN (p<0.001), 4°C/14d (p<0.05), and 4°C/28d (p<0.01, Table 1). Histopathology-score tended to increase with increasing 4°C storage duration. Histopathology-score was 4.4±1.2 in FRESH, and tended to be lower in 4°C/14d (p=0.2) and 4°C/28d (p=0.1) stored allografts. Histopathology-score was 12.1±0.7 in FROZEN, and was higher than FRESH (p<0.001), 4°C/14d (p<0.05), and 4°C/28d (p<0.05). Surface irregularity followed similar trends to overall histopathology-score, while vertical clefts, transverse clefts, and cloning were similar among FRESH, 4°C/14d, and 4°C/28d (p>0.7). Hypocellularity score in 4°C/14d and 4°C/28d stored allografts was higher than FRESH (each, p<0.001) and Non-OP (each, p<0.001), but lower than FROZEN (each, p<0.01, Table 1).

Cartilage Composition – Matrix Fixed Charge

Quantitatively, in addition to visualizing overall qualitative patterns, HE-µCT varied with allograft storage (p<0.001). HE-µCT value in Non-OP was similar to FRESH (p>0.6, Fig. 4.5). Matrix fixed charge tended to be lower in 4°C/14d and 4°C/28d stored allografts than FRESH (p=0.1, p=0.2, respectively), as indicated by ~20% increase in HE-µCT value (95%CI: 12-31% for 4°C/14d and 9-30% for 4°C/28d, Fig. 4.5). Matrix fixed charge was lowest in FROZEN; HE-µCT value in FROZEN was ~35% higher than Non-OP and FRESH (95% CI: 21-49%; each, p<0.001), and ~22% higher than 4°C/14d and 4°C/28d stored allografts (95% CI: 10-35%; each, p<0.05).

Collectively, HE-µCT analysis and Safranin-O histology indicated qualitatively that matrix fixed charge was similar between Non-OP and FRESH, slightly decreased in 4°C/14d and 4°C/28d, and further decreased in FROZEN allografts. Highest HE-µCT values (in red), which represent low matrix fixed charge, were present throughout the depth of FROZEN (Fig. 4.5B,L). Moderate HE-µCT values (in orange and yellow) were present in 4°C/14d and 4°C/28d (Fig. 4.5D-E,N-O), and low HE-µCT values (in blue), which represent high matrix fixed charge, was absent in FROZEN, and present in Non-OP and FRESH (Fig. 4.5A,C,K,M). Representative Safranin-O histology confirmed the mild loss of proteoglycans in 4°C/14d and 4°C/28d, and extensive loss in FROZEN versus Non-OP and FRESH allografts (Fig. 4.5F-J,P-T). Safranin-O score in Non-OP was similar to FRESH (p>1.0, Table 4.1). Safranin-O staining intensity was consistently high in FRESH (Saf-O=0, 5/7 allografts), moderate in 4°C/14d (Saf-O=1, 6/8), and weak in 4°C/28d (Saf-O=2, 5/8). Safranin-O score was 3.0±0.0, indicating absence of staining, in FROZEN, and higher than all storage treatments (p<0.001 vs. Non-OP and FRESH, p<0.01 vs. 4°C/14d and 4°C/28d, Table 4.1).

Biomechanical Function

Cartilage material stiffness varied with allograft storage (p<0.001), and with site (p<0.001), but without an interaction effect (p=0.3). Stiffness in Non-OP was similar to FRESH, 4ºC/14d, and 4ºC/28d at MFC (p>0.4, Fig. 4.6A) and at LT (p>0.8, Fig. 4.6B). Stiffness in FROZEN was lower than Non-OP, by ~80% at MFC (95%CI: 72-91%; p<0.01) and by ~95% at LT (95%CI: 93-97%; p<0.01). Stiffness in FROZEN also tended to be lower than FRESH, 4ºC/14d, and 4ºC/28d, by ~75% at MFC (95%CI: 57-92%; p=0.3 vs. FRESH, p=0.2 vs. 4ºC/14d, p=0.08 vs. 4ºC/28d),

and by ~94% at LT (95%CI: 88-99%; p=0.06 vs. FRESH, p=0.1 vs. 4ºC/14d, p=0.4 vs. 4ºC/28d).

Cartilage thickness varied with allograft storage (p<0.01), site (p<0.001), and with an interaction effect (p<0.05). Cartilage thickness was ~40% thinner (95%CI: 36-45%) at LT (0.72±0.21mm) than at MFC (1.78±0.54mm, Fig. 4.6). At MFC, cartilage thickness in 4°C/14d and 4°C/28d was ~45% thicker (95%CI: 28-65%; p<0.05) than FROZEN, but similar to FRESH (p>0.6).

Relationships of Cartilage Health and Cellularity

Indices of cartilage health, including gross-score, histopathology-score, matrix fixed charge, and cartilage stiffness, correlated significantly with cartilage cellularity (see Appendix Table 2). For MFC&LT, gross-score and histopathology-score correlated negatively with superficial cellularity (each, ρ^2=0.6, p<0.001, Fig. 4.7A-B). HE-µCT value, inversely related to matrix fixed charge, also correlated negatively with superficial cellularity (R^2=0.2, p<0.001, §4.7 Fig. 4.S2A), while cartilage stiffness correlated positively with overall cellularity (R^2=0.4, p<0.01, §Fig. 4.S2B). MFC and LT also exhibited similar trends to MFC&LT, except HE-µCT value versus superficial cellularity for MFC (R^2=0.1, p=0.1), and gross- and histopathology-score versus deep cellularity for MFC (ρ^2=0.3, p=0.1).

4.5   Discussion

The results demonstrate that reduced cellularity at the articular surface of osteochondral allografts resulting from various storage conditions was associated with poor structural, biological, and biomechanical repair outcomes at 12months in the goat. The cartilage of FRESH osteochondral allografts, representing the best condition of implant viability, had similar tissue properties to Non-OP site-matched cartilage, and the normal organization of chondrocytes provided sufficient biological remodeling capacity to sustain cartilage structure, matrix content, and mechanical properties (Figs. 2-6). In contrast, the cartilage of FROZEN osteochondral allografts, representing the worst condition of implant viability, was acellular (Fig. 4.2-4.3), and could not maintain biological homeostasis, and resulted in a tissue that was structurally deteriorated and mechanically soft (Figs. 4.4-4.6). Intermediately, the cartilage of 4°C-stored osteochondral allografts, stored at 4°C for 14days and 28days, were similar, and both were inferior to FRESH grafts, but marginally better than FROZEN grafts. Cellularity was reduced at the articular surface (Figs. 4.2-4.3), and accompanied by reduced matrix content (Fig. 4.5) and structural properties (Fig. 4.4, Table 4.1), suggesting that decreased cellularity, as a result of 4°C storage for 14-28days, detrimentally affects repair outcomes.

The associated decline in surface cellularity with overall tissue properties in retrieved osteochondral allografts suggests that 4°C-stored grafts are somewhat susceptible to tissue degeneration. Early signs of tissue structural deterioration in 4°C-stored grafts, including reduced proteoglycan content, increased surface irregularity, and poor graft-host cartilage integration, suggest inadequate biological remodeling capacity of the remaining chondrocytes to sustain long-term function. Although variable gross scores (range 1-9) and qualitatively diminished surface cellularity for

4°C-stored allografts were consistent with previous animal studies [21, 34, 37], the significant relationship between surface cellularity and repair outcomes reported here suggests that chondrocytes at the articular surface are vitally important to maintain tissue structure, composition, and function. Since surface chondrocyte viability during allograft storage appears to be a strong determinant of tissue properties, storage conditions that preserve surface viability is essential for long-term repair efficacy.

While reduced cellularity at the articular surface, as a result of 4°C storage, detrimentally affected repair efficacy in the goat, translation to the clinical situation may be dependent on several factors. The goat provided a useful large animal model for studying osteochondral defect repair because it exhibits limited intrinsic healing [10]; however, the 8mm diameter defects in the current study were larger than the critical defect size of 6mm for goats [16], although smaller than that commonly found in humans [10]. Measuring cellularity within 100μm from the surface distinguished a region of cartilage in the goat vulnerable to cell death. In human cartilage, cells within an absolute distance from the surface may be affected, or this vulnerable region may scale relative to cartilage thickness. Although chondrocyte viability was similar between human [32, 33] and caprine [22, 27] osteochondral tissue following clinically established 4°C storage conditions, *in vivo* repair efficacy in humans may be related to an absolute minimum number of cells rather than overall/regional cell density important in the goat model. Thus, evidence of reduced repair outcomes with increasing storage in this report suggests that 4°C storage duration should be limited prior to allograft implantation in patients.

Consistently good repair outcomes at MFC with FRESH allografts may be due to normal chondrocyte density *in vivo*, maintaining homeostasis of the osteochondral unit. Appropriate zonal chondrocyte organization in FRESH allografts provided

biological remodeling to preserve the articular surface, sustain bulk tissue properties, and prevent progressive deterioration to graft failure (Figs. 2-6). Such maintenance of bulk tissue properties allows cartilage to function normally by supporting joint loads through pressurizing interstitial fluid [2], and preventing excessive tissue deformation and fluid exudation into the underlying subchondral bone plate, which would accelerate cartilage and bone degeneration. While cartilage integrity was preserved, most allografts (irrespective of storage treatment) contained subchondral bone cysts at 12months; investigating the possible interactions between cartilage and bone during repair may be critical to osteochondral repair efficacy. Variable gross and cartilage histopathology results with FRESH allografts at LT may be due to inherent differences between the MFC and LT sites (i.e. cartilage thickness, loading environment), and are consistent with clinical results suggesting that patellofemoral lesions have worse clinical outcomes than femoral condyle lesions [4, 9, 18, 40]. However, some repair outcomes (i.e. cartilage stiffness and matrix content) were similar at MFC and LT, suggesting that the tissue is functioning normally, and these repair outcomes were consistent with maintenance of surface cellularity. Thus, cartilage repair outcomes for FRESH allografts serve as a good baseline from which to compare the outcomes of 4°C-stored grafts.

Osteochondral allograft insertion parameters reported here were generally below the levels to cause significant cell death [19, 31, 39], and were consistent with previous human cadaveric [6, 31] and animal [15] studies. While chondrocyte viability, especially at the articular surface, is generally inversely related to impact stress [19, 31, 39, 41], the relationship between long-term performance and insertion parameters may also be dependent of other factors. Repetitive trauma causes chondrocyte death, even if each impact is below the threshold for cartilage injury [8].

In the present study, low gross and histological scores, in association with decreased surface cellularity, for two grafts, which required many more taps than average (~100 taps, §4.7 Fig. 4.S3), may suggest that tap number is important to consider. The decreased performance of these grafts suggests that preserving chondrocyte viability during insertion is also critically important to long-term repair efficacy.

Examining chondrocyte organization by depth following cartilage repair treatments and its association with repair efficacy may help elucidate a critical number of functional chondrocytes within the appropriate cartilage zone(s). Superficial zone cellularity, $\geq 18 \times 10^6$cells/cm^3, was essential to maintain gross structural scores ≤ 3, which would characterize most FRESH allografts (5/7). Thus, most 4°C stored allografts, with ~15×10^6cells/cm^3 in the superficial zone, did not have sufficient surface cellularity to maintain cartilage structure. In addition, the strong correlation of overall cellularity with cartilage function suggests the importance of maintaining cellularity throughout the depth of cartilage. However, since early tissue degeneration is evident at the articular surface, maintenance of superficial zone cellularity may be a crucial contributor to repair efficacy.

The inferior repair outcomes from 4°C-stored versus fresh allografts suggest that alternative storage protocols that preserve the chondrocyte viability, especially in the superficial zone are needed to improve long-term repair efficacy. Shorter storage duration (<7days) that more closely mimics the FRESH condition is one potential option. Alternatively, osteochondral allograft storage at 37°C may be one option to support long-term viability of chondrocyte *in vitro*, especially at the articular surface [5, 22, 27]. Grafts stored at 37°C may also have better biological performance than 4°C-stored grafts, by providing lubricating molecules such as proteoglycan-4 (PRG4) to maintain a low-friction articulating surface [35, 36]. Since some 4°C-stored

allografts maintained relatively high surface cellularity, an alternative biological marker of superficial zone health may be useful to screen grafts prior to implantation. PRG4-secreting function of allografts appears to be maintained *in vivo* based on its state after storage [29]; thus, PRG4 secretion may be a useful predictor of suitable graft tissue. The long-term efficacy of allografts stored in conditions to enhance superficial zone chondrocyte viability (i.e. 37°C storage) remains to be elucidated.

4.6   Acknowledgments

Table 4.1: Histopathology and cartilage degeneration scores. [a]Gross score (0-9) was determined by assigning a score of 0-3 (with 0 representing normal, and 3 representing severe degeneration) for the following gross morphological characteristics: surface texture, defect filling/size, and graft-recipient cartilage integration [13]. [b]Histopathology score (0-15) was determined by assigning a score of 0-2 or 0-3 (with 0 representing normal, and 2 or 3 representing severe degeneration) for the following histologic characteristics: surface irregularity (0-2), vertical clefts into the transitional or radial zone (0-2), transverse clefts (0-2), cloning (0-3), hypocellularity (0-3), and Safranin-O staining (0-3) [38]. See §4.7 Table 4.S1 for scores at MFC and LT sites. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. FROZEN; #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs. Non-OP; $$p<0.05$, $$$p<0.01$, $$$$p<0.001$ vs. FRESH, %$p<0.05$ vs. 4°C/14d

MFC & LT	Non-OP	FRESH	4°C/14d	4°C/28d	FROZEN
n	15	7	8	8	7
Gross[a]	$0.4 \pm 0.1^{***}$	$4.0 \pm 0.8^{**}_{\#\#\#}$	$3.4 \pm 0.7^{***}_{\#\#\#}$	$5.0 \pm 0.8^{*}_{\#\#\#}$	7.9 ± 0.8
Surface Texture	$0.4 \pm 0.1^{***}$	$1.3 \pm 0.4_{\#}$	$1.1 \pm 0.3^{*}_{\#}$	$1.5 \pm 0.4_{\#\#}$	2.6 ± 0.3
Defect Filling/Size	$0.0 \pm 0.0^{***}$	$0.7 \pm 0.4^{**}$	$0.1 \pm 0.1^{***}$	$1.0 \pm 0.4^{*\%}_{\#\#}$	2.4 ± 0.4
Cartilage Integration	$0.0 \pm 0.0^{***}$	$2.0 \pm 0.2^{*}_{\#\#\#}$	$2.1 \pm 0.4_{\#\#\#}$	$2.5 \pm 0.2_{\#\#\#}$	2.9 ± 0.1
Histopathology[b]	$2.4 \pm 0.3^{***}$	$4.1 \pm 1.4^{***}$	$5.5 \pm 0.6^{**}_{\#\#\#}$	$6.3 \pm 0.5^{*\$}_{\#\#\#}$	12.4 ± 0.8
Surface Irregularity	$0.8 \pm 0.1^{**}$	$0.7 \pm 0.4^{**}$	1.3 ± 0.2	1.5 ± 0.2	1.9 ± 0.1
Vertical Clefts	$0.4 \pm 0.1^{*}$	0.7 ± 0.3	$0.4 \pm 0.2^{**}$	0.5 ± 0.3	1.6 ± 0.3
Transverse Clefts	$0.1 \pm 0.0^{*}$	0.1 ± 0.1	$0.0 \pm 0.0^{**}$	0.3 ± 0.2	1.0 ± 0.4
Cloning	$0.1 \pm 0.0^{***}$	$1.3 \pm 0.4_{\#\#\#}$	$1.1 \pm 0.3_{\#\#}$	$1.4 \pm 0.3_{\#\#\#}$	2.0 ± 0.3
Hypocellularity	$0.1 \pm 0.0^{***}$	$0.3 \pm 0.3^{***}$	$1.5 \pm 0.3^{*\$\$\$}_{\#\#\#}$	$1.3 \pm 0.3^{*\$\$}_{\#\#\#}$	3.0 ± 0.0
Safranin-O	$1.0 \pm 0.2^{***}$	$1.0 \pm 0.2^{***}$	$1.3 \pm 0.3^{**}$	$1.4 \pm 0.3^{**}$	3.0 ± 0.0
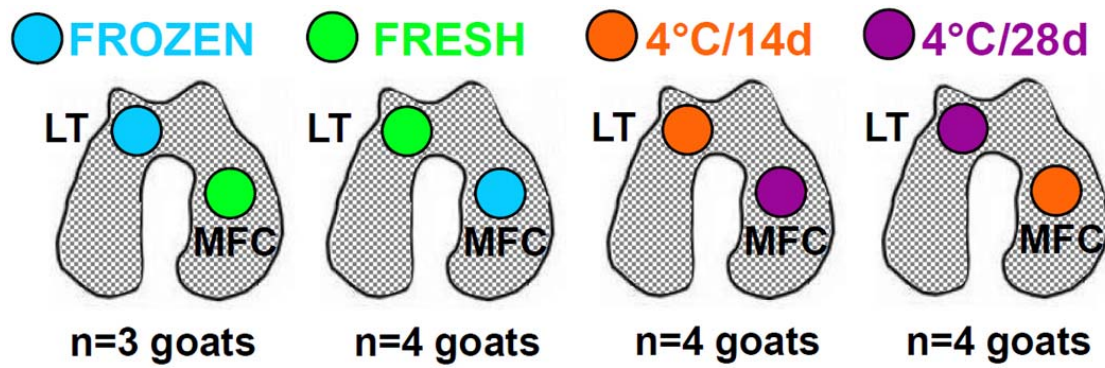
Figure 4.1: Schematic diagram of implanted allografts with various storage treatments.
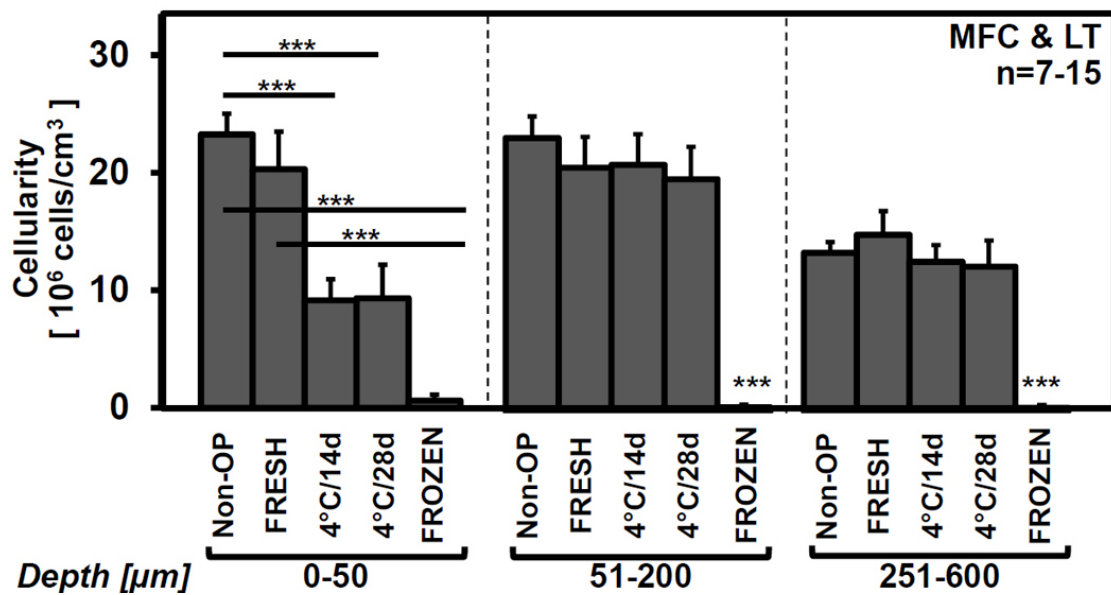
Figure 4.2: Effect of *in vivo* allograft storage on cellularity at selected (0-50, 51-200, 201-600μm) depths from the articular surface in retrieved osteochondral allografts after 12months. Mean±SEM. ***p<0.001. See §4.7 Figure 4.S1 for detailed cellularity by depth.
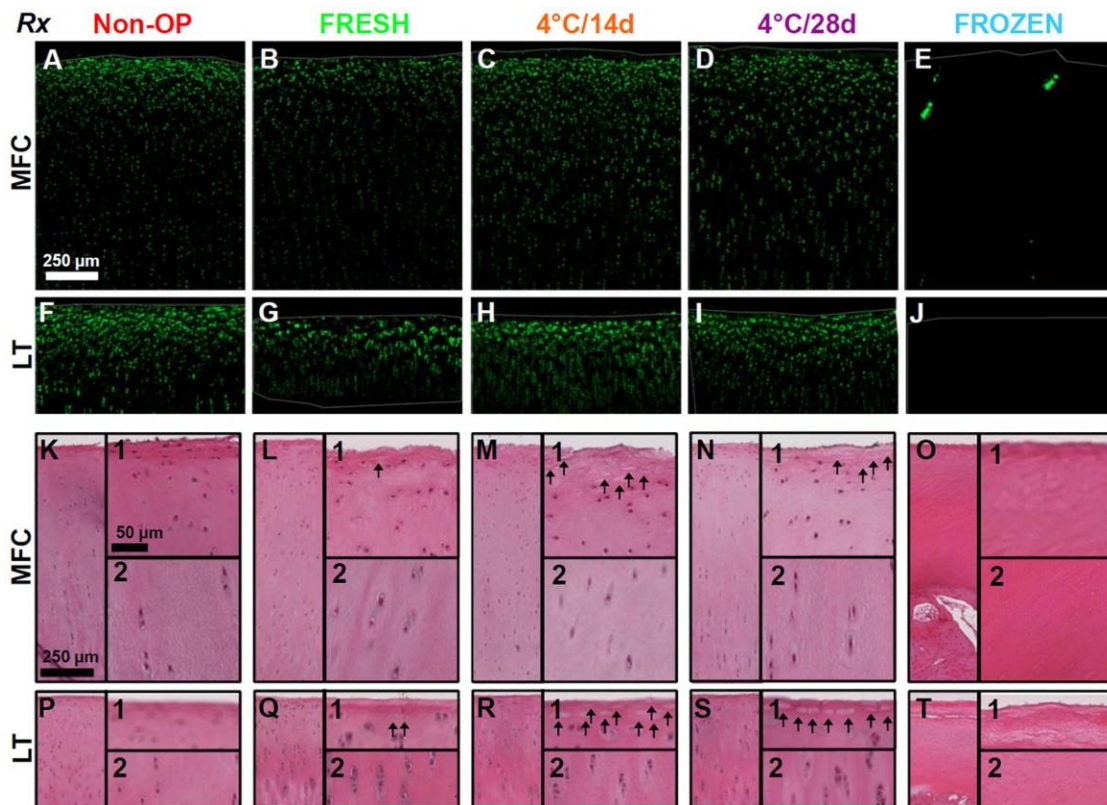
Figure 4.3: Qualitative cellularity analysis in retrieved allografts. Representative (**A-J**) confocal and (**K-T**) H&E histology images of vertical profile view throughout the depth of articular cartilage of retrieved osteochondral allografts at (**A-E,K-O**) MFC and (**F-J,P-T**) LT sites after 12months *in vivo*. H&E histology images depict the full-profile and zoomed regions of the (1) surface, and (2) deep zone. Thin gray lines denote the cartilage surfaces. Arrows indicate empty cellular lacunae.
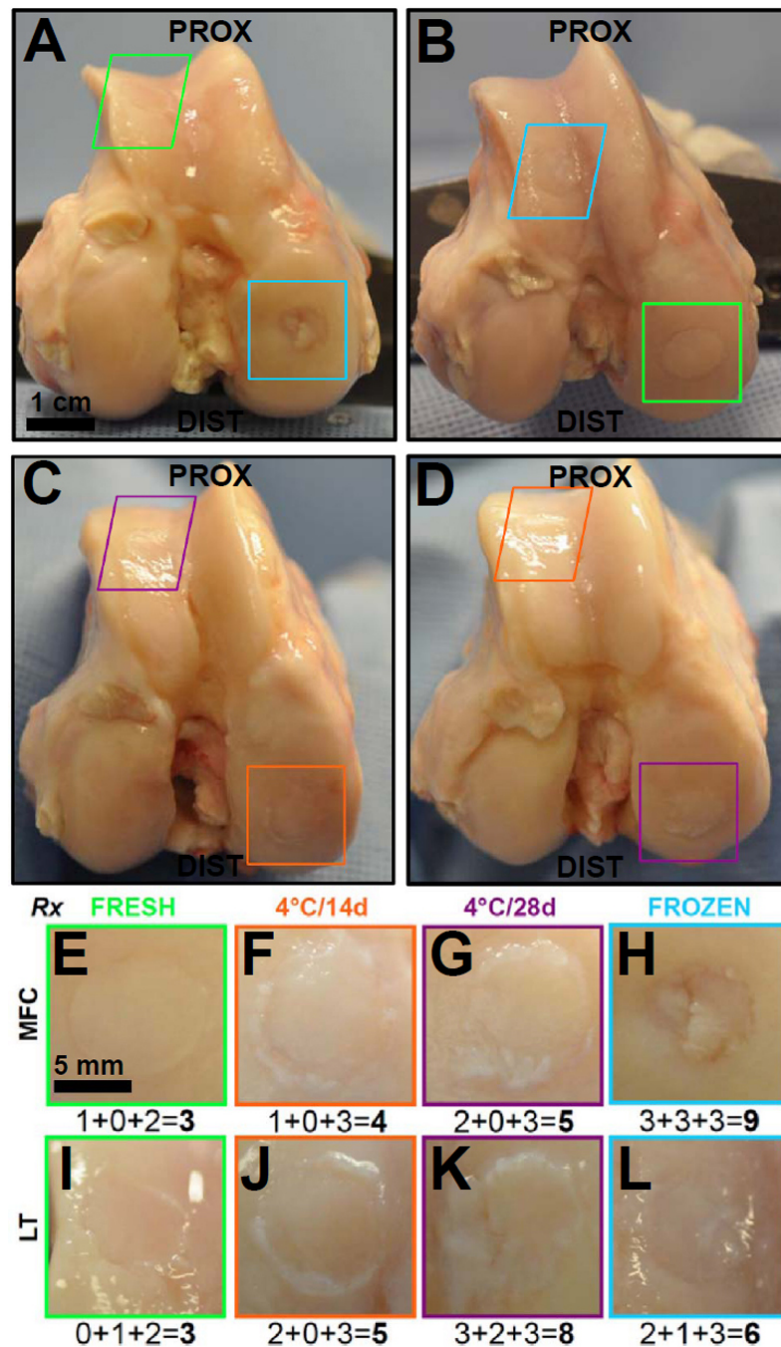
Figure 4.4: Structural analysis in retrieved allografts. Gross macroscopic images of representative (**A-D**) knee joints, and (**E-L**) individual retrieved FROZEN (blue), FRESH (green), 4°C/14d (orange), and 4°C/28d (purple) allografts at (**E-H**) MFC and (**I-L**) LT sites after 12months *in vivo*. Numbers in the lower right corner indicate the gross score assigned to the repair.
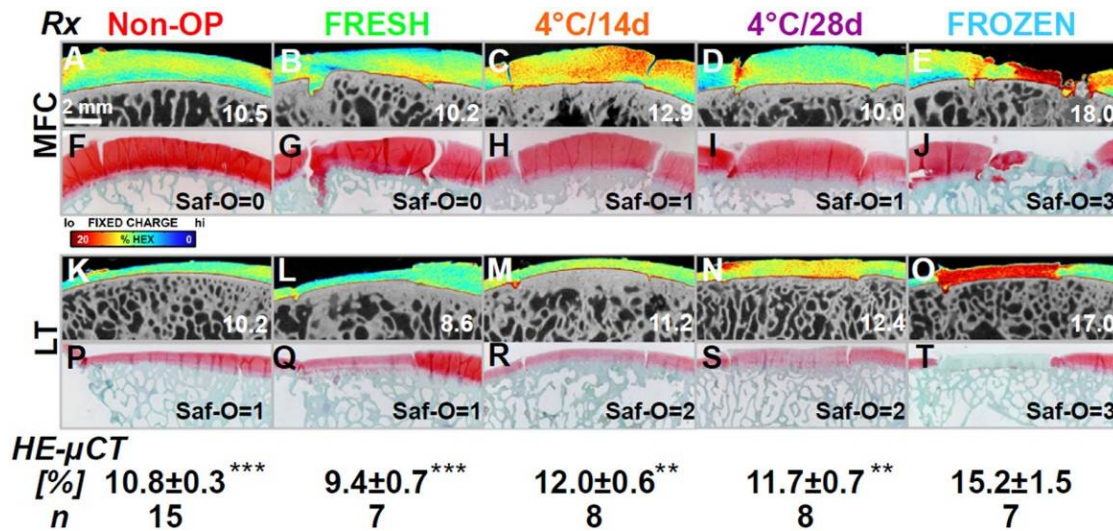
Figure 4.5: Analysis of matrix fixed charge in retrieved allografts. Representative (**A-E,K-O**) color maps of HE-μCT analysis and corresponding (**F-J,P-T**) Safranin-O histology of retrieved osteochondral allografts at (**A-J**) MFC and (**K-T**) LT sites after 12months *in vivo*. Values in lower right corner correspond to the score (HE-μCT value or Safranin-O) of the representative image. Mean±SEM. **p<0.01, ***p<0.001 vs. FROZEN.
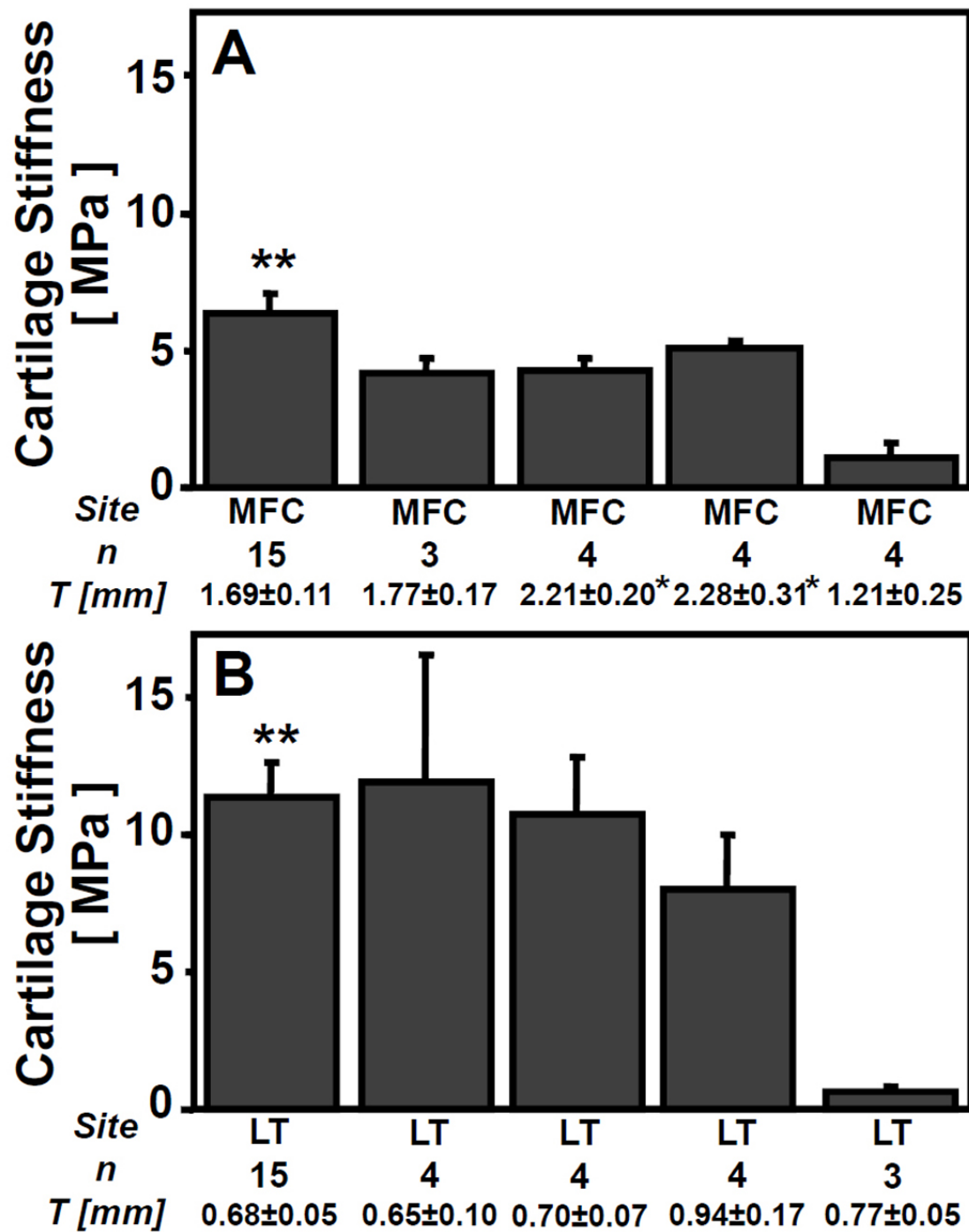
Figure 4.6: Effect of *in vivo* allograft storage on cartilage stiffness and thickness (T) in retrieved osteochondral allografts at (**A**) MFC and (**B**) LT sites after 12months. Mean±SEM. *p<0.05, **p<0.01 vs. FROZEN.
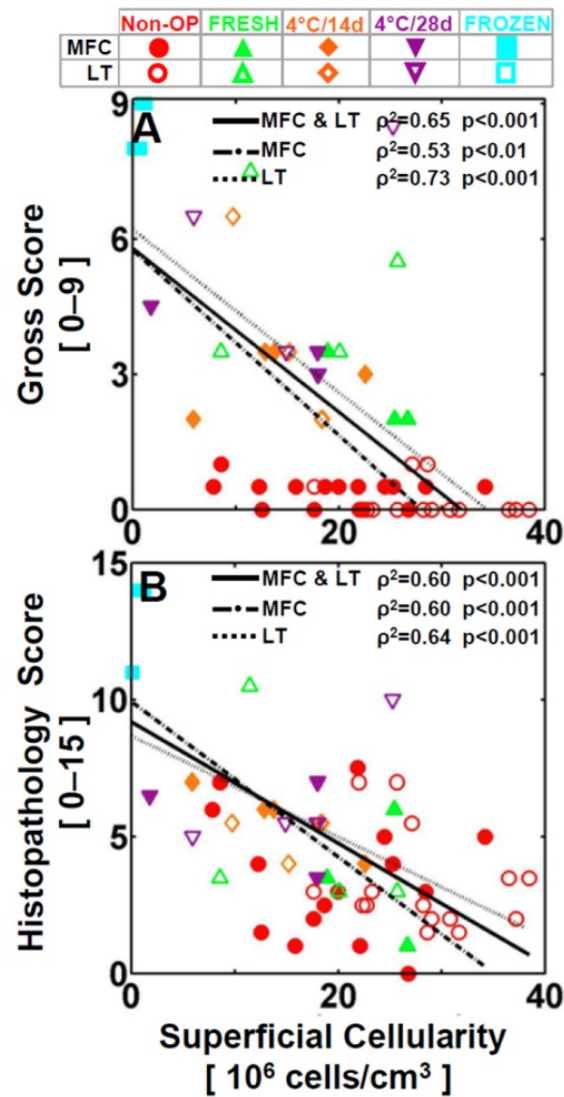
Figure 4.7: Relationship between cellularity and cartilage structure. Spearman's rank correlation of (**A**) gross score and (**B**) histopathology score vs. superficial cellularity for MFC (filled) and LT (open). Data points correspond to individual Non-OP (red circles), FRESH (green triangles), 4°C/14d (orange diamonds), 4°C/28d (purple inverted triangles), and FROZEN (blue squares) allograft retrievals. Significance (p) and regression coefficients (ρ^2) were determined.

4.7   Supplement

Supplementary tables and figures, cited in the text, report metrics at individual sites, for MFC and LT. For detailed gross and histopathology scores see Table 4.S1. For additional regression analysis see Table 4.S2 and Fig. 4.S2. For cellularity by depth from the surface see Fig. 4.S1. For impaction outliers see Fig. 4.S3.

Table 4.S1: Detailed gross and histopathology scores at MFC and LT. Mean±SEM, * vs. FROZEN, # vs. Non-OP, $ vs. FRESH, % vs. 4°C/14d

MFC	Non-OP	FRESH	4°C/14d	4°C/28d	FROZEN
n	15	3	4	4	4
Gross	$0.4 \pm 0.1^{***}$	$2.7 \pm 0.3^{**}_{\#\#\#}$	$3.5 \pm 0.4^{*}_{\#\#\#}$	$4.0 \pm 0.6_{\#\#\#}$	8.5 ± 0.3
Surface Texture	$0.4 \pm 0.1^{***}$	$0.7 \pm 0.2^{**}$	$1.1 \pm 0.1^{***}$	$1.3 \pm 0.1^{*}$	2.8 ± 0.1
Defect Filling/Size	$0.0 \pm 0.0^{***}$	$0.5 \pm 0.3^{***}$	$0.3 \pm 0.1^{***}$	$0.4 \pm 0.1^{***}$	2.8 ± 0.1
Cartilage Integration	$0.0 \pm 0.0^{***}$	$0.3 \pm 0.3^{***}$	$1.6 \pm 0.2^{\$\$}_{\#\#\#}$	$1.9 \pm 0.2^{\$\$}_{\#\#\#}$	3.0 ± 0.0
Histopathology	$3.5 \pm 0.6^{***}$	$3.5 \pm 1.4^{**}$	$5.8 \pm 0.6^{\$}_{\#}$	$6.5 \pm 1.2^{\$}_{\#}$	13.3 ± 0.8
Surface Irregularity	1.0 ± 0.2	0.8 ± 0.6	1.3 ± 0.3	1.0 ± 0.4	1.8 ± 0.3
Vertical Clefts	$0.6 \pm 0.1^{*}$	0.3 ± 0.2	0.5 ± 0.2	$0.5 \pm 0.5^{*}$	1.8 ± 0.3
Transverse Clefts	$0.5 \pm 0.1^{**}$	$0.3 \pm 0.3^{*}$	$0.4 \pm 0.1^{*}$	0.3 ± 0.3	1.4 ± 0.4
Cloning	$0.2 \pm 0.1^{***}$	$1.2 \pm 0.3^{*}_{\#\#\#}$	$0.9 \pm 0.2^{***}_{\#}$	$1.5 \pm 0.4^{\%}_{\#}$	2.4 ± 0.2
Hypocellularity	$0.4 \pm 0.1^{***}$	$0.3 \pm 0.2^{***}$	$1.6 \pm 0.3^{\$\$}_{\#\#}$	$1.1 \pm 0.3^{*\$}_{\#}$	3.0 ± 0.0
Safranin-O	$0.7 \pm 0.2^{**}$	$0.5 \pm 0.3^{*}$	1.3 ± 0.5	$2.1 \pm 0.3^{\$}$	3.0 ± 0.0

LT	Non-OP	FRESH	4°C/14d	4°C/28d	FROZEN
n	15	4	4	4	3
Gross	$0.2 \pm 0.1^{***}$	$5.0 \pm 1.0_{\#\#\#}$	$3.5 \pm 1.1_{\#\#\#}$	$5.4 \pm 1.3_{\#\#\#}$	5.8 ± 1.2
Surface Texture	$0.2 \pm 0.1^{**}$	$1.4 \pm 0.4_{\#}$	1.1 ± 0.4	$1.8 \pm 0.6_{\#}$	1.8 ± 0.4
Defect Filling/Size	$0.0 \pm 0.0^{***}$	$1.4 \pm 0.4_{\#\#\#}$	$0.5 \pm 0.4^{**}_{\$}$	$1.3 \pm 0.5^{\%\%}_{\#\#\#}$	1.8 ± 0.6
Cartilage Integration	$0.0 \pm 0.0^{***}$	$2.3 \pm 0.3_{\#\#\#}$	$2.0 \pm 0.4_{\#\#\#}$	$2.4 \pm 0.2_{\#\#\#}$	2.2 ± 0.3
Histopathology	$3.5 \pm 0.6^{***}$	5.0 ± 1.8	$5.1 \pm 0.4_{\#}$	$6.5 \pm 1.2_{\#}$	10.7 ± 0.9
Surface Irregularity	0.4 ± 0.2	0.4 ± 0.4	0.9 ± 0.2	1.0 ± 0.4	1.7 ± 0.2
Vertical Clefts	0.2 ± 0.1	0.6 ± 0.5	0.0 ± 0.0	0.5 ± 0.5	0.7 ± 0.3
Transverse Clefts	0.2 ± 0.1	0.3 ± 0.3	0.1 ± 0.1	0.3 ± 0.3	0.8 ± 0.6
Cloning	$0.1 \pm 0.1^{*}$	$2.0 \pm 0.4_{\#\#\#}$	$1.8 \pm 0.1_{\#\#\#}$	$1.5 \pm 0.4_{\#}$	1.5 ± 0.3
Hypocellularity	$0.1 \pm 0.1^{***}$	$0.3 \pm 0.3^{***}$	$0.9 \pm 0.3^{\$}_{\#\#}$	$1.1 \pm 0.3^{\$}_{\#\#}$	3.0 ± 0.0
Safranin-O	$1.5 \pm 0.2^{**}$	1.5 ± 0.2	1.5 ± 0.8	2.3 ± 1.2	3.0 ± 0.0

Table 4.S2: Regression analysis of indices of cartilage health versus cartilage cellularity. Spearman rank (ρ^2) and linear (R^2) regression coefficients; significance (p); slope (m)

MFC & LT

Cellularity [10^6 cells/cm³]	Gross			Histopathology			HE-μCT [%]			Stiffness [MPa]		
	ρ^2	p<	m	ρ^2	p<	m	ρ^2	p<	m	ρ^2	p<	m
Superficial	0.65	0.001	–0.18	0.60	0.001	–0.22	0.20	0.001	–0.11	0.16	0.01	0.20
Middle	0.41	0.01	–0.13	0.48	0.001	–0.23	0.14	0.01	–0.11	0.37	0.001	0.36
Deep	0.39	0.01	–0.12	0.47	0.001	–0.20	0.09	0.05	–0.10	0.38	0.001	0.44
Overall	0.49	0.001	–0.18	0.52	0.001	–0.27	0.15	0.01	–0.13	0.40	0.001	0.45

MFC

Cellularity [10^6 cells/cm³]	Gross			Histopathology			HE-μCT [%]			Stiffness [MPa]		
	ρ^2	p<	m	ρ^2	p<	m	ρ^2	p<	m	ρ^2	p<	m
Superficial	0.53	0.01	–0.21	0.60	0.001	–0.28	0.10	0.08		0.16	0.01	0.11
Middle	0.45	0.05	–0.34	0.66	0.001	–0.56	0.06	0.20		0.37	0.001	0.20
Deep	0.41	0.05	–0.47	0.58	0.001	–0.73	0.05	0.25		0.38	0.001	0.33
Overall	0.52	0.01	–0.44	0.67	0.001	–0.66	0.08	0.13		0.20	0.001	0.26

LT

Cellularity [10^6 cells/cm³]	Gross			Histopathology			HE-μCT [%]			Stiffness [MPa]		
	ρ^2	p<	m	ρ^2	p<	m	ρ^2	p<	m	ρ^2	p<	m
Superficial	0.73	0.001	–0.18	0.64	0.001	–0.18	0.30	0.01	–0.14	0.12	0.06	
Middle	0.44	0.05	–0.13	0.45	0.05	–0.20	0.26	0.01	–0.15	0.26	0.01	0.34
Deep	0.29	0.11		0.31	0.10		0.14	0.05	–0.12	0.27	0.01	0.37
Overall	0.54	0.01	–0.17	0.47	0.01	–0.23	0.26	0.01	–0.17	0.30	0.01	0.42
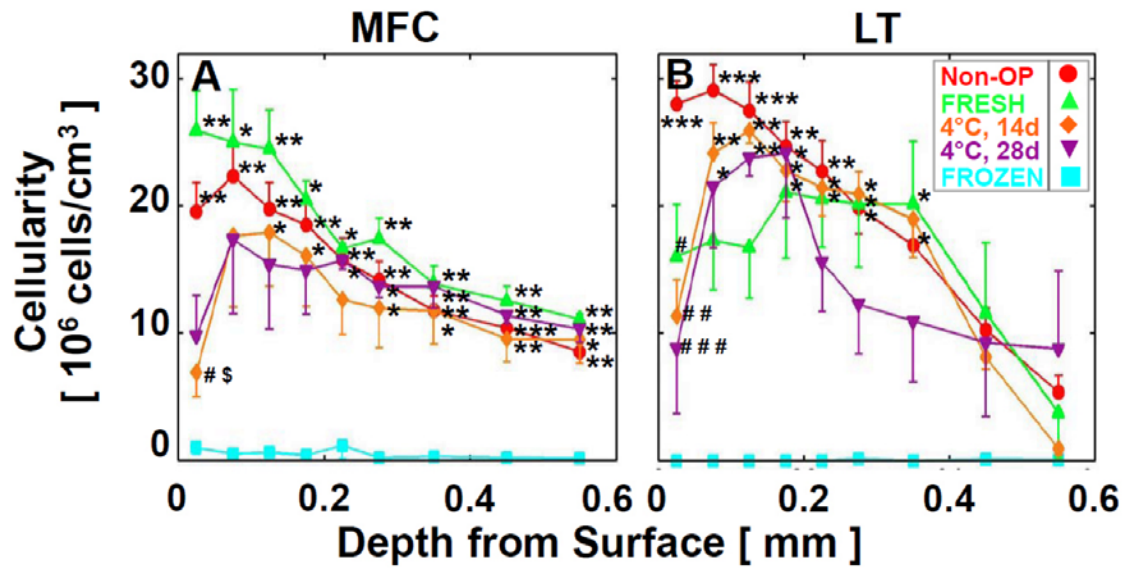
Figure 4.S1: Effect of *in vivo* allograft storage on cellularity by depth from the articular surface (0-0.6mm) in retrieved osteochondral allografts at (A) MFC and (**B**) LT sites after 12months. Mean±SEM, n=3-15. *p<0.05, **p<0.01 vs. FROZEN # vs. Non-OP, $ vs. FRESH.
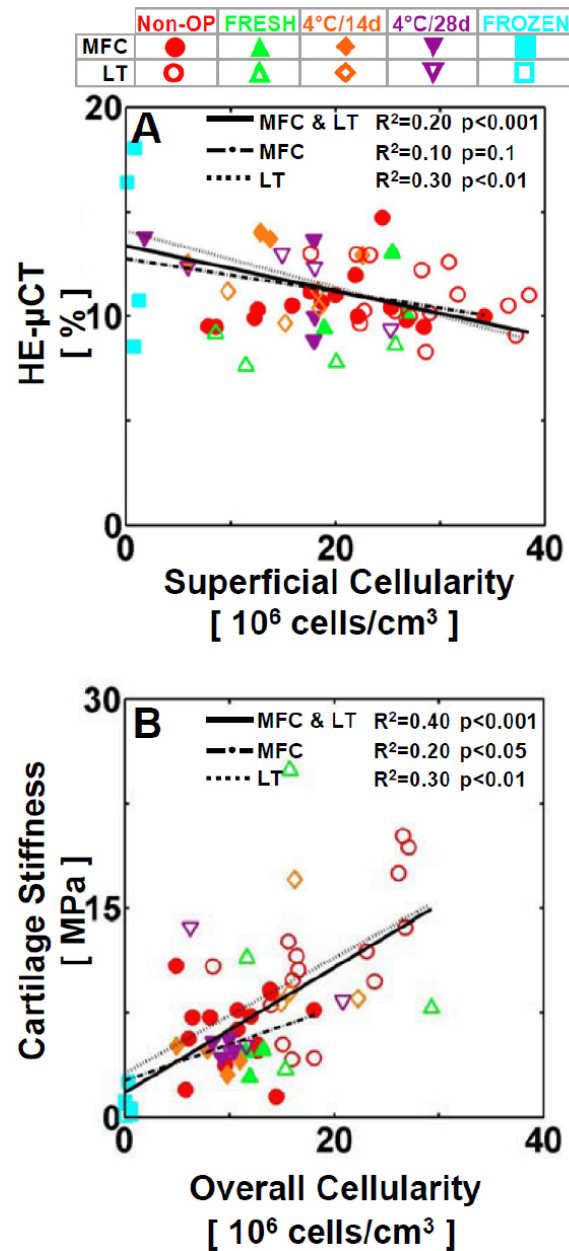
Figure 4.S2: Parametric regression analysis of the relationship between (**A**) HE-μCT values vs. superficial cellularity and (**B**) cartilage stiffness vs. overall cellularity for MFC (filled) and LT (open). Data points correspond to individual Non-OP (red circles), FRESH (green triangles), 4°C/14d (orange diamonds), 4°C/28d (purple inverted triangles), and FROZEN (blue squares) allograft retrievals. Significance (p) and regression coefficients (R^2) were determined.

Rx	FRESH AVG	FRESH Outlier	4°C/28d AVG	4°C/28d Outlier
Gross Image				
Force [N]	201±46	246	210±36	205
Taps [ ]	39±25	96	45±29	112
Gross Score	2.7±0.3	8	5.0±0.8	9
Histology Score	4.1±1.4	12	6.3±0.5	9

Figure 4.S3: Effect of *in vivo* allograft insertion for average and outlying samples on structural scores after 12months. Mean±SD.

4.8   References

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CHAPTER 5:

PRG4 SECRETION OF STORED OSTEOCHONDRAL ALLOGRAFTS IS ASSOCIATED WITH *IN VIVO* REPAIR EFFICACY AND PRESERVATION OF THE ARTICULAR SURFACE IN THE GOAT AT 12 MONTHS

5.1 Abstract

Objective: Reduced cellularity at the articular surface, resulting from 4°C storage, is associated with variable long-term outcomes in the goat. Examining chondrocyte function at the vulnerable articular surface, by production of the lubricant molecule proteoglycan-4 (PRG4) from superficial zone chondrocytes, may identify biomarkers in pre-clinical models that predict allograft biological performance. The hypothesis of this study was that decreased PRG4 secretion in stored allografts is associated with decreased chondrocyte cellularity and poor *in vivo* repair outcomes. **Design:** The effect of allograft storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on PRG4 secretion after (i) storage, and (ii) 12months *in vivo* was assessed in adult goats. Relationships between PRG4 secretion after 12months *in vivo* and repair outcomes, including cartilage surface cellularity, structural scores, and biomechanical function, were assessed by linear regression. **Results:** PRG4 secretion decreased with increasing 4°C storage duration, as PRG4 secretion in 4°C/14d was similar to FRESH, but ~25x higher than FROZEN and in 4°C/28d was similar to FROZEN, tended to be

~67% lower than 4°C/14d, and was ~75% lower than FRESH. After 12months *in vivo*, PRG4 secretion of allografts appeared to be maintained based on its state after storage. Indices of *in vivo* repair efficacy correlated significantly with subsequent PRG4 secretion in retrieved allografts at 12months. **Conclusions:** PRG4-secreting function of cartilage following osteochondral allografting serves as a useful index of long-term function. This study confirms the utility of PRG4 as a biomarker indicative of the normal biology of the cartilage surface function and its relationship to long-term *in vivo* repair outcomes.

5.2   Introduction

Osteochondral allografts (OCA), used to repair large articular cartilage defects, ideally restores the cartilage surface in a structurally and functionally appropriate manner. However, long-term allograft efficacy depends on the presence of viable and functional chondrocytes to maintain cartilage homeostasis and preserve joint function. In current clinical practice, fresh-stored OCA are used after storage at 4°C for 15-43days, which compromises chondrocyte viability, and has resulted in some short-term clinical improvement [4, 12, 15, 28]. However, in long-term animal models of such fresh-stored OCA, repair outcomes are variable [13, 21, 24], and associated with surface cellularity [21]. Since surface cellularity appears to be an important predictor of long-term graft efficacy, but has considerable variation during prolonged storage intervals, functional predictors of allograft biological performance are needed to identify suitable graft tissue for implantation.

Restoration of cartilage surface biology and function following cartilage repair would be advantageous for long-term biological performance. In animal models, effectiveness of cartilage repair by osteochondral grafts, assessed by mechanical stiffness of cartilage, is associated with cartilage surface properties, including articular surface deviation relative to the native osteochondral structure [3], gross macroscopic structure, articular surface irregularity, and superficial zone cellularity [21, 23]. Identifying determinants of cartilage surface function may indicate early-stage cartilage deterioration and lead to future improvement in the application of OCA. Thus, examining chondrocyte function at the important, and vulnerable articular surface, and its relationship to *in vivo* repair efficacy may be useful to further understand the factors necessary for OCA success or failure.

Biosynthesis of the lubricant molecule proteoglycan-4 (PRG4), which is

secreted by chondrocytes in the superficial zone [27], contributes to the maintenance of cartilage homeostasis and the preservation of normal joint function. PRG4 biosynthesis exhibits mechanosensitivity [5, 16-19, 29], indicates cartilage surface health [8, 9, 25], and may be a useful biomarker in cartilage repair, as it depends on the presence of chondrocytes at the articular surface and represents normal surface biology. In short-term animal studies, PRG4 secretion of OCA, after storage frozen or usage fresh, was a sensitive measure of cartilage surface function and appeared to be maintained *in vivo* based on its state after storage [22]. However, further investigations into the relationship between long-term OCA efficacy, in association with variable cellularity, and PRG4 secretion are needed to evaluate this potential biomarker.

Thus, the hypothesis of this study was that decreased PRG4 secretion in stored OCA is associated with decreased chondrocyte cellularity and poor repair outcomes. The specific aims were to determine the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on PRG4 secretion after (i) storage, and (ii) 12months *in vivo* in adult goats, and to assess the association between PRG4 secretion and *in vivo* repair efficacy.

5.3   Materials and Methods

Experimental Design

Experiment 1: OCA Storage. The effects of FROZEN, FRESH, and 4°C OCA storage on the rate of PRG4 secretion from adult goat cartilage was determined. After storage at -70°C (FROZEN), or at 4°C for 3d (FRESH), 14d (4°C/14d), or 28d (4°C/28d), cartilage was isolated and incubated in medium supplemented with 10 ng/mL TGF-β1 to stimulate PRG4 secretion [26]. Cartilage-conditioned medium (CM, n=2-3 animals) was analyzed for PRG4 by ELISA.

Experiment 2: *In Vivo* OCA. The effects of transplantation of stored OCA (FROZEN, FRESH, 4°C/14d, 4°C/28d) after 12months *in vivo* on subsequent PRG4 secretion was determined using tissue from a previous study [21]. Portions of cartilage from FROZEN, FRESH, 4°C/14d, and 4°C/28d OCA at medial femoral condyle (MFC) and lateral trochlea (LT) sites, along with site-matched regions of non-operated (Non-Op) joints and cartilage at the lateral femoral condyle (LFC) site, were retrieved after 12months *in vivo* (n=3-8 at each site) and analyzed for PRG4 secretion. CM was analyzed for PRG4 by ELISA and Western blot. Relationships between PRG4 secretion and measures of in vivo repair efficacy previously reported [21], including superficial zone cellularity, gross- and histopathology- score, and cartilage stiffness, were assessed by linear regression.

OCA Storage

OCA were prepared from adult Boer goats (2-4yo) and stored prior to implantation. Some osteochondral samples were stored FROZEN at -70°C for 10days. Other fragments were stored at 4°C in tissue culture medium (low-glucose Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 0.1mM non-essential amino acids,

2mM L-glutamine, 25μg/mL L-ascorbic acid, and antibiotics-antimycotics (100U/mL penicillin, 100μg/mL streptomycin, and 0.25μg/mL fungizone, PSF) for 3d (FRESH), 14d (4°C/14d), or 28d (4°C/28d). For PRG4 secretion in Exp 1, FROZEN, FRESH, 4°C/14d, and 4°C/28d donor OCA were stored as LFC fragments (each, n=4-6 joints) from both knees of adult Boer goats (n=2-3 animals) described previously [21]. Following storage, cartilage explants (d=5mm) were used for subsequent analysis.

Cartilage Culture and Conditioned Medium (CM)

Cartilage from OCA was removed from the bone and incubated in media containing TGF-β1 to stimulate chondrocyte PRG4 secretion [26], and collected for subsequent analysis, as previously described [22]. Following storage or retrieval, cartilage explants, with the articular surface intact, were incubated in medium (low-glucose Dulbecco's modified Eagle's medium, 10mM HEPES buffer, 0.1mM non-essential amino acids, 0.4mM L-proline, 2mM L-glutamine, 25μg/mL L-ascorbic acid, and PSF) containing 10ng/mL TGF-β1 and 0.01% bovine serum albumin for 15-20days at 37°C in an atmosphere with 5% CO_2. For PRG4 secretion of OCA donors in Exp 1, CM was pooled over 15days. For PRG4 secretion of OCA retrievals in Exp 2, CM was pooled over 20days. Medium (surface area/volume/day=0.75cm^2/mL/d) was replaced every 2-4days, and CM was collected for subsequent analysis.

Quantification and Characterization of PRG4 in CM

The CM collected from cartilage cultures was analyzed for PRG4 by indirect ELISA and/or Western blot, as previously described [10, 22]. Briefly, for ELISA, portions of CM was diluted serially, adsorbed, and then reacted with monoclonal antibody 3-A-4 (MD Bioproducts, St. Paul, MN), horseradish peroxidase-conjugated

secondary antibody, and ABTS substrate. Rates of PRG4 secretion were calculated by normalizing the total mass of PRG4 in the CM to cartilage surface area and incubation duration. Briefly, for Western blot, portions of CM were pooled, separated by horizontal agarose gel electrophoresis, transferred to polyvinyidene difluoride membrane, and probed with 3-A-4, with ECL-Plus detection. Luminescence from the membrane was digitized with STORM 840 Imaging System (Molecular Dynamics, Fairfield, CT).

Statistical Analysis

Data are presented as mean±SEM, with $p < 0.05$ considered significant. In Exp 1, the effect of OCA storage (FROZEN, FRESH, 4°C/14d, 4°C/28d) on PRG4 secretion was assessed ANOVA. In Exp 2, the effects of OCA storage (Non-Op, FROZEN, FRESH, 4°C/14d, 4°C/28d) on PRG4 secretion was assessed by ANOVA. ANOVAs were followed by Tukey *post hoc* tests to determine which experimental group means differed from each other. Also in Exp 2, for Non-Op, the effect of site (MFC+LT, LFC) and knee (Op vs. Non-Op) on PRG4 secretion was determined by *t*-test. Statistical analyses were performed using Systat 10.2 (Systat Software, Chicago, IL).

The relationship between PRG4 secretion and retrieved cartilage properties of OCA after 12months *in vivo* were assessed by regression. Cartilage properties from retrieved stored OCA, including superficial zone cellularity, gross- and histopathology- score, and cartilage stiffness, were reported previously as measures of in vivo repair efficacy [21]. Gross- and histopathology-scores were correlated with PRG4 secretion by non-parametric Spearman's rank method. Cartilage stiffness and cellularity in the superficial zone were correlated with PRG4 secretion by linear

regression. Coefficients of determination, R^2 (parametric) and ρ^2 (nonparametric), are reported for significant relationships ($p<0.05$).

5.4 Results

Experiment 1: OCA Storage

PRG4 secretion by cartilage explants isolated from stored donor OCA varied with storage ($p<0.001$). After FRESH OCA storage, PRG4 secretion over 15d was 3.07 ± 0.14 $\mu g/(cm^2 \cdot d)$, and ~30x higher than FROZEN ($p<0.01$, Fig. 5.1), which was 0.10 ± 0.01 $\mu g/(cm^2 \cdot d)$. After 4°C/14d OCA storage, PRG4 secretion was similar to FRESH ($p>0.5$), and ~25x higher than FROZEN ($p<0.05$, Fig. 5.1). After 4°C/28d OCA storage, PRG4 secretion was ~75% lower than FRESH ($p<0.05$), tended to be lower by ~67% than 4°C/14d ($p=0.06$), and was similar to FROZEN ($p>0.6$, Fig. 5.1).

Experiment 2: In Vivo OCA

After 12months *in vivo*, PRG4 secretion from cartilage of the retrieved OCA varied with storage ($p<0.001$). PRG4 secretion in Non-Op was 2.94 ± 0.26 $\mu g/(cm^2 \cdot d)$, similar to FRESH ($p>0.5$, Fig. 5.2A), and similar at MFC, LT, and LFC sites ($p>0.7$). In addition, at LFC, PRG4 secretion was similar between Non-Op and Op joints ($p>0.5$, Fig. 5.2A). In contrast, PRG4 secretion after FROZEN storage was only 0.66 ± 0.37 $\mu g/(cm^2 \cdot d)$, and was markedly reduced by ~78% versus Non-Op ($p<0.001$) and ~70% versus FRESH ($p<0.05$), and similar to 4°C/14d ($p>0.3$) and 4°C/28d ($p>0.7$, Fig. 5.2A). PRG4 secretion after 4°C/14d and 4°C/28d storage was reduced versus Non-Op, by ~45% ($p<0.05$) and ~55% ($p<0.01$), respectively, and similar to FRESH ($p>0.7$, $p>0.3$, respectively, Fig. 5.2A).

Qualitatively, the CM contained a component consistent with PRG4, as evidenced by a major immunoreactive band on Western blot with monoclonal antibody 3-A-4. This immunoreactive band was evident for CM from Non-Op and FRESH, slightly lighter from 4°C/14d and 4°C/28d, and lighter from FROZEN OCA.

The localization of immunoreactive material on Western Blot and the difference in band intensities between samples from different groups (Fig. 5.2B) appeared consistent with the differences in PRG4 secretion determined by ELISA (Fig. 5.2A).

Indices of *in vivo* repair efficacy, including gross-score, histopathology-score, superficial cellularity, and cartilage stiffness correlated significantly with subsequent PRG4 secretion in retrieved OCA at 12months. For MFC&LT, gross-score and histopathology-score correlated negatively with PRG4 secretion (each, $\rho^2=0.5$, $p<0.001$, Fig. 5.3A-B). PRG4 secretion correlated positively with superficial cellularity ($R^2=0.3$, $p<0.001$, Fig. 5.3C) and cartilage stiffness ($R^2=0.1$, $p<0.05$, Fig. 5.3D). MFC and LT also exhibited similar trends to MFC&LT, except cartilage stiffness versus PRG4 secretion for LT ($R^2=0.1$, $p=0.1$, Fig. 5.3D).

5.5   Discussion

These results demonstrate that decreased PRG4 secretion in stored OCA storage was associated with decreased surface cellularity and poor structural, biological, and biomechanical *in vivo* repair outcomes at 12months in the goat. After 12months *in vivo*, the cartilage of adult goat osteochondral samples that were stored FRESH secreted PRG4 into culture medium at a high rate both after storage (Fig. 5.1) and after retrieval (Fig. 5.2). In contrast, the cartilage of FROZEN OCA released PRG4 at a low rate after retrieval *in vivo* (Fig. 5.2), but slightly higher than that of FROZEN-stored cartilage prior to implantation (Fig. 5.1). Intermediately, PRG4 secretion tended to decrease with increasing 4°C storage duration; the cartilage of 4°C/14d OCA released PRG4 at a rate similar to FRESH and higher than FROZEN after storage and 12months *in vivo*, whereas the cartilage of 4°C/28d released PRG4 at a rate similar to FROZEN and lower than FRESH after storage and 12months *in vivo*. Reduced PRG4 secretion after retrieval was associated with reduced superficial cellularity, along with diminished structural and biomechanical properties of cartilage. Thus, the PRG4-secretion of the cartilage of OCA is maintained *in vivo* in a manner that reflects its state after *in vitro* storage before implantation, and may be a useful predictor of long-term *in vivo* performance.

The use of the adult goat model to study correlates and markers of *in vivo* allograft performance, in association with OCA storage, involved the consideration of a number of issues. To probe the PRG4 secretory responses, the *in vitro* culture conditions were chosen to vigorously stimulate PRG4 secretion [26]; the PRG4 secretion rates during *in vitro* culture may differ from those *in vivo*. Assessing PRG4 secretion and other retrieved cartilage properties in adjacent regions within the repair tissue allowed for such integrative analysis between biological and structural

outcomes, without compromising the individual metrics.

The associated decline in PRG4 secretion with surface cellularity and overall tissue properties in retrieved osteochondral allografts suggests that PRG4 secretion may be a useful marker of biological function following cartilage repair, and that 4°C stored allografts have diminished biological function versus fresh allografts. Early signs of tissue structural deterioration in these 4°C stored allograft, including reduced proteoglycan content, increased surface irregularity, and poor graft-host integration [21], suggest that 4°C storage compromises the ability of chondrocytes to sustain tissue homeostasis and prevent subsequent cartilage deterioration. Biomarkers, including PRG4/lubricin, have been extensively studied for osteoarthritis [2, 11], and were first introduced for osteochondral allograft repair by our group [22]. While translation to the clinical situation is still unknown, this study confirms the utility of PRG4 as a biomarker indicative of the normal biology of the cartilage surface function and its relationship to long-term *in vivo* repair outcomes.

PRG4 secretion rates after osteochondral allograft storage and retrieval in the goat were consistent with previous studies, and extend the past work to incorporate clinically relevant 4°C storage conditions. PRG4 secretion appeared to be maintained in non-operated cartilage and fresh allografts after 12months *in vivo* versus previous shorter term *in vivo* durations of 6months [22]; although PRG4 secretion from frozen allografts was slightly higher in this report than at 6months. "Cartilage flow" of adjacent host tissue, where peripheral cartilage begins to curve and push into the graft region [3, 6, 7], may have contributed to the elevated PRG4 secretion rates in frozen allografts, since allograft margins were ill-defined and bulk tissue was inhomogeneous [21]; however, the substantially lower PRG4 secretion rates in frozen allograft retrievals versus non-operated cartilage suggests that normal surface functions of

adjacent host cartilage were also compromised during frozen allograft associated cartilage failure. Thus, the PRG4-secreting function of cartilage following repair appears to serve an index of long-term function and clinical efficacy.

The diminished PRG4 secretion in prolonged 4°C stored versus fresh allografts after storage, in association with inferior *in vivo* repair outcomes, suggests that PRG4 secretion could be useful for screening allografts prior to implantation in order to improve allograft efficacy. Short 4°C storage durations that closely mimic the fresh condition may eliminate the need for screening allografts prior to implantation; however, such short durations make acquisition and distribution of these allografts logistically challenging. Alternatively, storage conditions, such as storage at physiological temperature of 37°C, which support long-term viability of chondrocytes in vitro, especially at the articular surface [1, 14, 20], may provide higher quality osteochondral tissue by maintaining the articular surface integrity and sustaining PRG4 secretion of surface chondrocytes. However, since some 4°C stored allograft maintained relatively high surface cellularity [21], in conjunction with high PRG4 secretion rates, despite prolonged storage durations, selecting these grafts based using PRG4 secretion after storage as a predictor of allograft performance may improve overall repair outcomes in patients.

5.6   Acknowledgments
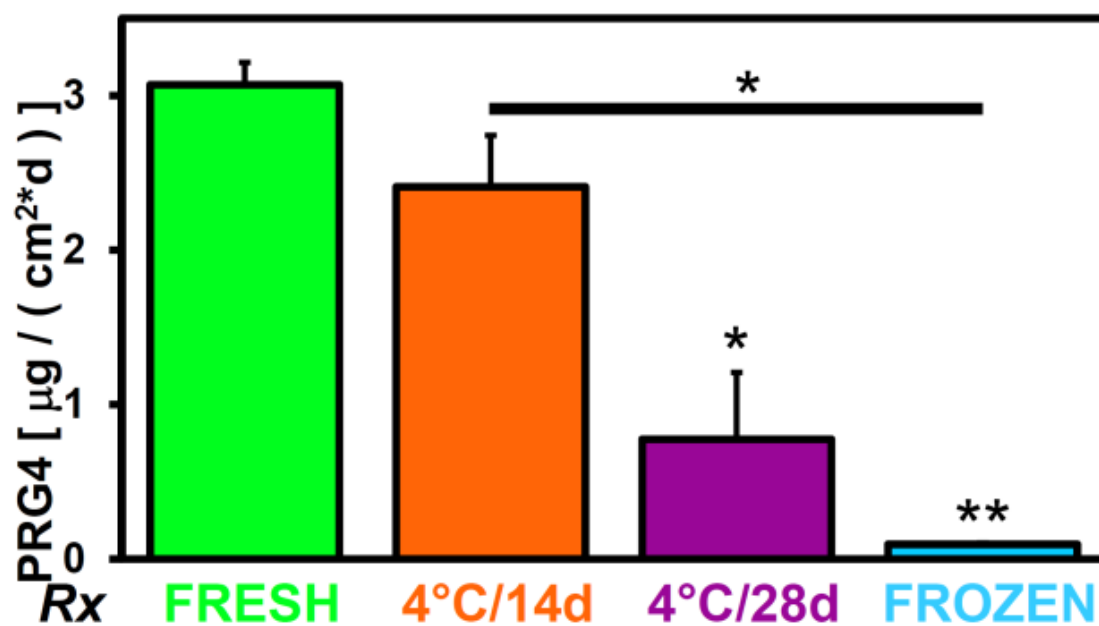
Figure 5.1: Effect of OCA storage on rate of PRG4 secretion from OCA. n=2-3 animals, mean±SEM. *p<0.05,**p<0.01 vs. FRESH.

Figure 5.2: Effect of transplantation of OCA after 12months *in vivo* on subsequent rate of PRG4 secretion in culture. (**A**) Quantification by ELISA, n=7-15, mean±SEM. *p<0.05, **p<0.01 ***p<0.001 vs. Non-Op. (B) Characterization by Western blot.

Figure 5.3: Relationship between rate of PRG4 secretion in culture and cartilage properties of retrieved OCA after 12months *in vivo*. (A) Gross-score, (B) histopathology-score, (C) superficial cellularity, (D) cartilage stiffness. Data points correspond to individual Non-Op (red circles), FRESH (green triangles), 4°C/14d (orange diamonds), 4°C/28d (purple inverted triangles), and FROZEN (blue squares). Significance (p) and regression coefficients (ρ^2 for A-B, R^2 for C-D) were determined.

5.7 References

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CHAPTER 6:

CHONDROCYTE VIABILITY IS HIGHER AFTER PROLONGED STORAGE AT 37°C THAN AT 4°C FOR OSTEOCHONDRAL GRAFTS

6.1 Abstract

Background: Osteochondral allografts are currently stored at 4ºC for 2-6 weeks before implantation. At 4ºC, chondrocyte viability, especially in the superficial zone, deteriorates starting at 2 weeks. Alternative storage conditions could maintain chondrocyte viability beyond 2 weeks, and thereby facilitate increased graft availability and enhanced graft quality.

Purpose: Determine effects of prolonged 37°C storage compared to traditional 4ºC storage on chondrocyte viability and cartilage matrix content.

Study Design: Controlled Laboratory Study

Methods: Osteochondral samples from humeral heads of adult goats were analyzed (**i**) fresh, or after storage in medium for (**ii**) 14d at 4°C including 10% FBS, (**iii**) 28d at 4°C including 10% FBS, (**iv**) 28d at 37°C without FBS, (**v**) 28d at 37°C including 2% FBS, or (**vi**) 28d at 37°C including 10% FBS. Portions of samples were analyzed by microscopy after LIVE/DEAD® staining to determine chondrocyte viability and density, both *en face* (to visualize the articular surface) and vertically (overall and in superficial, middle, and deep zones). The remaining cartilage was analyzed for sulfated-glycosaminoglycan and collagen.

Results: 37°C storage maintained high chondrocyte viability compared to 4°C storage. Viability of samples after 28d at 37°C was ~80% at the cartilage surface *en face*, ~65% in the superficial zone, and ~70% in the middle zone, which was much higher than ~45%, ~20%, and ~35%, respectively, in 4°C samples after 28d, and slightly decreased from ~100%, ~85%, and ~95%, respectively, in fresh controls. Cartilage thickness, glycosaminoglycan content, and collagen content were maintained for 37°C and 4°C samples compared to fresh controls.

Conclusion: 37°C storage of osteochondral grafts supports long-term chondrocyte viability, especially at the vulnerable surface and superficial zone of cartilage.

Clinical Relevance: Storage of allografts at physiological temperature of 37ºC may prolong storage duration, improve graft availability, and improve treatment outcomes.

6.2   Introduction

Osteochondral allografting is used for the repair of large articular cartilage defects by restoring mature, hyaline cartilage in a biologically, structurally, and functionally appropriate manner. Young, active patients with large ($> 2cm^2$) chondral or osteochondral defects are suitable candidates for osteochondral allografting [2, 12, 25, 34]. In addition, osteochondral allografting is performed when other surgical techniques, such as marrow stimulation and cell transplantation, have failed previously or are contraindicated [12, 34]. Osteochondral allografts have been used for treatment of focal cartilage defects [33, 40, 59], osteochondritis dissecans [19, 23], as well as post-traumatic, osteonecrotic, and bipolar lesions in the knee [6, 11, 41]. Overall, usage of such allografts has resulted in clinical success rates exceeding 75% [6, 10, 11, 14, 19, 23, 41]. For transplantation of allografts, as with any allogenic organ or tissue, potential disease transmission and immunogenicity are important considerations [2, 14, 25, 26, 34]; however, the limited availability of suitable grafts, due in part to storage duration, restricts widespread application [25, 26]. Currently, fresh graft tissue is stored at 4ºC to accommodate federal regulations that require tissue banks to screen and test donor tissue for infectious diseases prior to implantation [1]. Such testing typically requires 14 days [26, 33, 59]; grafts are used after prolonged storage on average 24 days after harvest (range 15-43 days) [26, 33, 40, 59]. During short-term storage at 4°C, cartilage can be maintained in a biologically viable, but quiescent state, one in which chondrocyte metabolism is lower than normal [5, 9]. However, during prolonged storage at 4°C, chondrocyte viability deteriorates with a substantial decrease by 28 days and with a decrease to as low as 40% by 14 days [5, 15, 17, 46, 48, 53, 57, 58, 61]; such deterioration has led to recommended actions for a maximum duration of graft storage ("shelf-life") and an implantation

time within 30 days of harvest [33, 40]. Alternative storage conditions that maintain chondrocyte viability for a longer duration may lead to an increase in "shelf-life", and in turn, the availability of osteochondral allografts to surgeons and their patients.

Normally, chondrocyte metabolism contributes to cartilage homeostasis, and low chondrocyte viability within allografts is associated with tissue degeneration. Fresh osteochondral allografts, procured within 48 hours of donor death and implanted within 7 days, have high chondrocyte viability; retrieved grafts have contained viable chondrocytes up to 29 years after implantation [13, 30, 39, 42, 60]. Massive, frozen osteochondral allografts have been used in oncologic reconstructions after limb-salvaging resection of joint tissue due to tumors [35, 37, 38]. In contrast to fresh allografts, frozen grafts are devoid of viable chondrocytes, and specimens retrieved at 8 months to 5 years after reconstructive surgery generally display degenerate and acellular cartilage [20, 21]. Graft efficacy may be improved by implanting tissue with high chondrocyte viability to better maintain tissue composition, structure, and function [16, 20, 21].

During the routine storage of osteochondral donor tissue at 4ºC, death of chondrocytes increases with storage duration, especially in the superficial zone of cartilage. Certain 4ºC storage solutions can improve chondrocyte viability, while maintaining cartilage matrix content. Compared to fresh controls, grafts stored at 4°C for 28 days in a variety of storage solutions (i.e. lactated Ringers solution, serum-free culture medium) had lower chondrocyte viability, especially in the superficial zone [5, 15, 46-48, 53, 57, 58, 61], while maintaining cartilage glycosaminoglycan (GAG) content [5, 48]. During 4°C storage, supplementation of culture medium with fetal bovine serum (FBS) improved chondrocyte viability in osteochondral grafts, especially in the superficial region, compared to other storage solutions described

above, although viability was still lower than that in fresh controls [5, 46, 48, 53]. In addition, pre-equilibration of medium with 5% CO_2, which maintains physiological pH near 7.4, improved chondrocyte viability in grafts compared to equilibration with ambient air, which is traditionally used during 4°C storage [17]. Since chondrocytes exhibit zone-specific functions [3], assessing depth related variations in chondrocyte viability may help to understand graft performance. The loss of viable chondrocytes, especially in the superficial zone, may contribute to graft degeneration and subsequent failure, since early stage cartilage deterioration is evident at the articular surface. Graft efficacy may benefit from improving chondrocyte viability by modifications to storage conditions.

Incubation of osteochondral samples at physiological temperature may prevent storage-associated chondrocyte death and also maintain GAG content in the cartilage. In osteochondral cultures retained on their natural bone support, the chondrocytes remained viable, and the cartilage showed no signs of degeneration, even after prolonged storage (3-6 wks) at 37°C in medium with various concentrations of serum [18, 32]. Cartilage explants and osteochondral fragments incubated at 37°C in medium with different serum concentrations resulted in varying amounts of GAG deposition [7, 27, 28, 32, 54, 55]; immature cartilage explants in medium with 10-20% FBS incubated for 2-3 weeks maintained GAG content [28, 43]. Thus, the objectives of this study were to determine the effects of prolonged 37ºC storage compared to traditional 4ºC storage on the (1) chondrocyte viability, especially in the superficial zone, and (2) cartilage matrix content in fresh, goat osteochondral grafts.

6.3   Materials and Methods

Osteochondral Harvest

Osteochondral cores (n=65) were harvested from seven humeral heads of four mature (3-4 yr) male Boer goats. Caprine shoulders with intact joint capsules were obtained from a USDA licensed vendor and used within 48 hours of sacrifice. Under sterile conditions, joints were isolated and osteochondral cores were harvested by, first, scoring the cartilage surface with a 3.0 mm dermal punch and, second, coring with a custom 3.2 mm bit. The cartilage was kept hydrated throughout the procedure with copious amounts of phosphate-buffered saline (PBS) supplemented with antibiotics-antimycotic, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone (PSF). Prior to storage, each sample was thoroughly rinsed with PBS+PSF, and the bone was trimmed to a 4:1 bone to cartilage thickness ratio. For all samples, the subchondral bone was not analyzed quantitatively by LIVE/DEAD® staining because qualitatively it contained only dead cells and no viable cells, and this subchondral bone viability did not vary with storage condition.

Storage Conditions

Six storage conditions were analyzed (Table 6.1). Samples were either analyzed fresh (group i) or after storage for 14 or 28 days (groups ii-vi). Storage medium consisted of minimum essential medium Eagle with additives (10 μg/mL ascorbic acid, 2 mM L-glutamine, and PSF) with 0% FBS, but including 0.01% bovine serum albumin, 2% FBS, or 10% FBS. Samples stored at 4°C with 10% FBS for 14 or 28 days (groups ii and iii) were placed in glass vials with medium pre-equilibrated to 5% CO_2 and sealed tightly. Samples stored at 37°C with 0%, 2%, or 10% FBS (groups iv, v, and vi) were incubated for 28 days in 24-well plates in an

atmosphere with 5% CO_2. At the end of storage, 4°C samples were cut in half with a single edged razor blade (using a custom stainless steel jig that was pre-cooled on ice), gradually warmed at room temperature for 30 minutes, and then incubated at 37°C for an additional 48 hours to bring samples to standard culture conditions. At the end of storage, 37°C samples were cut in half (using a jig warmed at 37°C), and then incubated for an additional 48 hours. Medium, 1.5 mL/sample, was changed three times per week, a relative medium volume and replenishing frequency that were sufficient to keep cartilage viable during prolonged 37°C storage of osteochondral fragments [32].

Chondrocyte Viability

All samples were analyzed for live and dead cells using LIVE/DEAD® (Molecular Probes, Inc., Eugene, OR) staining, fluorescence microscopy, and image processing. Each half-core sample was stained by incubation in medium containing 2.7 μM calcein AM and 5.0 μM ethidium homodimer-1 for 15 minutes at 37°C. Metabolically active cells permit calcein AM to enter through the intact plasma membrane where the dye is cleaved by cytoplasmic esterases yielding green fluorescence [56]. In contrast, ethidium homodimer-1, which is membrane impermeable, binds to DNA of membrane-compromised cells yielding red fluorescence [45]. Samples were fluorescently imaged along a vertical profile using a 10x Plan Fluor objective lens (NA = 0.3; Nikon), a microscope (Eclipse TE300, Nikon, Melville, NY), an arc lamp, a G-2A (for "dead" images; Nikon) or B-2A (for "live" images; Nikon) filter cube, and a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI) to obtain images indicating live and dead cells. The articular cartilage with the surface intact was then removed from the bone, and the articular

surface was imaged *en face*. For each 8-bit grayscale image with 1600 x 1200 pixels, the field of view was 1.2 x 0.9 mm^2.

The images were processed to determine the cartilage thickness and to count live and dead cells using a custom routine (MATLAB$^®$ v7.5 with Image Processing Toolkit, Mathworks, Natick, MA) adapted from previous studies [4]. Briefly, images were processed [24] by spatial filtering (5 x 5 Laplacian of Gaussian filter) to accentuate regions representative of cells, median filtering to suppress noise, and thresholding with a size criteria (greater than 40 or 15 μm^2 for live or dead cells, respectively) to localize cells. This method had ~90% sensitivity for both live and dead cells as determined from twelve images of six samples by manual counting of randomly cropped regions containing >100 cells. Chondrocyte viability was calculated as the percentage of live cells relative to the total number of cells. Cell density, the number of cells divided by the imaged cartilage area (cells/mm^2), was also determined for live, dead, and total cells. For vertical profiles, viability and cell densities were calculated for the overall cartilage thickness as well as zones of cartilage; superficial, middle, and deep zones were defined as the top 15%, the next 35%, and the remaining 50% of the cartilage thickness, respectively, based on pilot studies to identify zones of cartilage based on regional differences in cell morphology.

Cartilage Matrix Content

Some of the remaining half-cores (n=31) were analyzed for sulfated-glycosaminoglycan (GAG) and collagen (COL) content in the cartilage of samples, fresh after harvest or following storage (n=4-9). Full thickness cartilage was removed from the bone, weighed wet, and solubilized with proteinase K at 60°C for 16 h [62]. Portions of the digest were analyzed to quantify the content of GAG by the dimethylmethylene blue dye binding assay [22] and COL by p-

dimethyalaminobenzaldehyde binding for hydroxyproline [63]. The hydroxyproline content was converted to COL content using a mass ratio of 7.25 collagen to hydroxyproline [29, 44]. All biochemical measures were normalized to wet weight.

Statistical Analysis

Data are presented as mean ± SEM. The effects of storage condition on chondrocyte viability, live, dead, and total cell density, in addition to cartilage thickness, GAG and COL content, were determined by a one-way ANOVA, with joint as a random factor. Where significant differences were detected, Tukey post hoc comparisons were performed to determine the effects of (1) storage duration at 4°C (conditions i, ii, iii) and (2) storage condition at day 28 (conditions iii, iv, v, vi). For viability and density data, zone (superficial, middle, and deep) was considered a repeated measure, and a two-way repeated measures ANOVA was performed. Since percentages form binomial rather than normal distributions, an arcsine transformation was applied to normalize viability data before the statistical analyses [52]. Significance was set at $\alpha=0.05$ and all statistical analyses were performed using Systat 10.2 (Systat Software Inc., Richmond, CA).

6.4 Results

Qualitative analysis indicated that osteochondral samples stored at 37°C had higher chondrocyte viability than samples stored at 4°C, especially at the articular surface. *En face* (Fig. 6.1A-C) and vertical (Fig. 6.2A-C) profiles showed that fresh controls, immediately following harvest, contained primarily live cells and relatively few dead cells. After 28 days of storage, 4°C samples stored in medium with 10% FBS had a large proportion of dead cells, especially at the articular surface (Figs. 6.1G-I and 6.2G-I), whereas 37°C samples stored in medium with 0%, 2%, or 10% FBS showed a relatively large proportion of live cells at the articular surface (Figs. 6.1J-R and 6.2J-R). Vertical profiles also showed scattered cell death in deeper regions of the tissue with similar patterns for all storage conditions (Fig. 6.2D-R).

Quantitative analysis, for both e*n face* and vertical profiles, indicated that chondrocyte viability varied significantly with storage condition ($p < 0.001$, Fig. 6.3A-E). For vertical profiles, chondrocyte viability also varied with zone ($p < 0.001$), with a significant interaction between zone and storage condition ($p < 0.001$). Consistent with these findings, live and dead cell densities in *en face* and vertical images varied with storage condition ($p < 0.001$, except $p < 0.01$ for overall vertical), without effects on total cell density ($p = 0.1$, Fig. 6.3F-T). For vertical profiles, live, dead, and total cell density varied with zone ($p < 0.001$), with a significant interaction between zone and storage condition ($p < 0.05$).

Effects of Storage Duration at 4°C

During 4°C storage, quantitative analysis at the cartilage surface *en face* indicated that chondrocyte viability decreased markedly with increasing storage duration (Fig. 6.3A). *En face*, chondrocyte viability was initially high for fresh

controls at the cartilage surface, nearing ~100%. After 14 days of 4°C storage, viability tended to decrease by ~15% (p=0.2) compared to fresh controls. During 4°C storage, viability decreased by ~40% (between days 14 and 28, p<0.001), and by ~55% after 28 days (vs. fresh controls, p<0.001).

During 4°C storage, quantitative analysis of the vertical profile indicated that chondrocyte viability markedly decreased with increasing storage duration, most notably in the superficial zone, and to a lesser extent in the middle and deep zones (Fig. 6.3B-E). Chondrocyte viability was initially high for fresh controls in the superficial, middle, and deep zones, averaging 84%, 94%, and 88%, respectively. In the superficial zone, viability decreased by ~25% after 14 days (vs. fresh controls, p<0.05), by ~55% (between days 14-28, p<0.001), and by ~80% after 28 days (vs. fresh controls, p<0.001). In the middle and deep zones, viability was not significantly lower after 14 days (decreased by ~10% and ~5% vs. fresh controls, p=0.7 and p=1.0, respectively), but was markedly decreased by ~50% and ~30% (between days 14-28, p<0.01), and by ~60% and ~35% after 28 days (vs. fresh controls, p<0.001, p<0.01, respectively). Due to the similarity in chondrocyte viability between 4°C storage after 14 days and fresh controls, the effects of storage condition (37°C vs 4°C) on chondrocyte viability was examined after 28 days of storage.

Effects of 37°C vs 4°C Storage at Day 28

After 28 days of storage, quantitative analysis at the articular surface *en face* was consistent with the qualitative results described above and indicated that 37°C storage maintained chondrocyte viability more effectively than 4°C storage (Fig. 6.3A). After 28 days, *en face* chondrocyte viability was ~35% higher following 37°C

storage compared to 4°C storage (p<0.001). At 37°C, *en face* viability among 0-10% FBS samples did not vary significantly (p=1.0).

After 28 days of storage, quantitative analysis of the vertical profile indicated that the superficial and middle zones had chondrocyte viability patterns similar to the surface viewed *en face*, while deep zone chondrocyte viability was not significantly different between 37°C and 4°C storage. At 37°C, chondrocyte viability among 0-10% FBS samples was not significantly different in any zone (p=1.0). For 0-10% FBS samples stored at 37°C, viability was higher by ~45% in the superficial zone (p<0.01), and by ~35% in the middle zone (p<0.05), compared to samples stored at 4°C (Fig. 6.3B,C). In the deep zone, viability of 37°C and 4°C stored samples was not significantly different, decreasing to ~45-55% after 28 days of storage (p=1.0) (Fig. 6.3D). Overall, chondrocyte viability throughout the vertical profile after 28 days fell to ~40% after 4°C storage, but only to ~60% after 37°C storage (p=0.1, Fig. 6.3E).

After 28 days of storage, live and dead cell densities reflected the relative percentages above, while total cell density was not significantly different between 37°C and 4°C storage. Total cell density was highest at the articular surface *en face* (2,400 cells/mm^2) and in the superficial zone (2,200 cells/mm^2), moderate in the middle zone (1,500 cells/mm^2) and in the overall vertical profile (1,300 cells/mm^2), and lowest in the deep zone (1,000 cells/mm^2) (Fig. 6.3P-T). At the articular surface *en face*, fresh controls had a high live cell density of 2,400 cells/mm^2, and a low dead cell density of <100 cells/mm^2 (Fig. 6.3F,K). For 4°C samples, *en face* live cell density fell to 1,200 cells/mm^2, and dead cell density rose to 1,200 cells/mm^2 after 28 days, indicating ~50% less live cells in 4°C samples compared to fresh controls (p<0.001). For 37°C samples, live and dead cell densities at the articular surface *en face* were moderately affected by 28-day storage compared to fresh controls,

averaging a live cell density of 2,000 cells/mm^2 and a dead cell density of 400 cells/mm^2 (for live: p=0.7, for dead: p<0.05).

Stored osteochondral samples, at both 37°C and 4°C for 28 days, had similar cartilage matrix content, as determined by GAG and COL, compared to fresh controls (Table 6.2). Cartilage thickness, GAG content, and COL content did not vary with storage condition (p=0.2, p=0.2, p=0.7, respectively). GAG content of samples for all storage conditions and durations was maintained within ~20% of fresh controls (35 mg/g). COL content of 4°C samples stored for 14d and 28d varied by ~15% compared to fresh controls (118 mg/g); but COL content of 37°C samples stored for 28d remained within ~5% compared to fresh controls.

6.5   Discussion

This study examined the effects of 37°C and 4°C storage on chondrocyte viability and cartilage matrix content of fresh goat osteochondral samples. After 28 days, 37°C samples had higher chondrocyte viability compared to 4°C samples, specifically at the articular surface. 4°C samples had a significant decline in chondrocyte viability to ~50% at the cartilage surface *en face*, to ~20% in the superficial zone, and to ~40% in the middle zone (Figs. 1G-I, 2G-I, and 3A-C), whereas 37°C samples had higher viability in those regions, averaging ~80%, ~65%, and ~70%, respectively (Figs. 1J-R, 2J-R, and 3A-C). In the deep zone, viability was similar at ~45-55% following 37°C and 4°C storage (Figs. 1, 2, and 3D). Additionally, there were no significant differences in cartilage thickness and matrix (GAG, COL) content for 37°C samples, 4°C samples, and fresh controls (Table 6.2). These results indicate 37°C storage of osteochondral grafts supports long-term viability of chondrocytes, especially at the articular surface.

The use of goat osteochondral samples for studying allograft storage conditions involved consideration of a number of issues. The small size of cores allowed many samples to be harvested from each joint, with the cores still having a sample radius (r=1.6 mm) that was greater than the cartilage thickness (t=0.5-1.0 mm). Therefore, the shortest path of diffusion, through the cartilage thickness, would be the same for these small cores or larger samples. For this study, storage of osteochondral samples in 1.5 mL of medium provided a medium volume that was ~50 times the total volume of the sample (cartilage+bone), while previous studies stored donor tissue in medium ~10 times the total volume of the sample [48, 61].

Viability data of caprine tissue from this study were similar to viability data of human tissue following clinically established 4°C storage conditions. Consistent with

the results presented here, 4°C storage of human osteochondral grafts resulted in high chondrocyte death, especially at the articular surface [46, 48]. The metabolic properties of adult goat cartilage during storage have not been previously investigated, and may differ compared to human cartilage, especially at physiological temperature. However, adult goats can provide consistent normal cartilage compared to humans, where aging and degeneration can often be a factor. Therefore, viability data suggests that storage of adult goat osteochondral samples can be a reasonable model for adult human osteochondral samples for up to one month. However, differences in cartilage thickness between caprine and human tissue may affect the diffusion of nutrients to the deeper regions of cartilage. Thus, studies with human tissue are needed to confirm the present findings.

Chondrocyte viability results presented here after 28-day 4°C storage were consistent with previous studies. Overall full-thickness viability averaged 45-78% in medium including FBS for human [46, 48], bovine [53], canine [57], and ovine [58] models. Average viability through the vertical profile, determined by fluorescent microscopy of thin sections [53] (~45%) or by confocal microscopy [57] (~65%), were more similar to the results here (~40%). Results reported by flow cytometry[46, 58], which required digestion of the cartilage matrix, or averaging images at random locations parallel to the articular surface [48], were at the high end of the range above (67-78%). However, these methods do not account for regional variations and may not provide an accurate representation of viability; cells may be lost during digestion, and averages may be skewed by ineffective sampling (i.e. inconsistent locations between samples and greater frequency in less affected regions).

The results presented here extend the past work on chondrocyte viability after 4°C storage by assessing quantitative estimates of chondrocyte viability at the

articular surface *en face* and in morphologically analogous cartilage zones. Previously, cartilage was analyzed as three zones of equal thickness, an assumption that would overestimate the cellularity of the true superficial zone (33% vs. 15% of cartilage thickness), and reported lower viability in the top-third (~50%) compared to overall viability (~67%) after 28-day 4°C storage [48]. Division of the cartilage into superficial, middle, and deep zones makes it difficult to directly compare viability to those of past studies. In addition, previous studies reported high overall viability (averaging ~80-95%) after 14-day 4°C storage [5, 53, 57, 58, 61] and qualitatively described preferential cell death at the cartilage surface with increasing storage duration [5, 17, 47, 58]. In the present study, viability after 14-day 4°C storage (~80%) was consistent with previously reported values of overall viability. Previous studies may not have been sensitive to declines in morphologically defined superficial zone, which occurred in this study as early as 14 days after 4°C storage.

Long-term storage of osteochondral grafts at 37°C and 4°C may have opposing effects on chondrocyte viability and GAG metabolism; at 37°C, most chondrocytes are viable and matrix content can be modulated whereas at 4°C, most chondrocytes are dead and metabolically inactive. Following 28-day 4°C storage, osteochondral grafts and cartilage explants had no change in GAG content compared to baseline controls; however, chondrocytes exhibited reduced GAG metabolism following 4°C storage compared to baseline controls when GAG synthesis was measured at physiological temperature [5, 9, 47, 61]. During cartilage explant culture, cellular outgrowth has been observed during incubation for 4-6 weeks at 37°C in the presence of 10-20% FBS, particularly from deep zone chondrocytes [8, 36]; such outgrowth may also occur during 37°C storage of osteochondral grafts and affect subsequent *in vivo* performance. In addition, following 28-day 37°C storage, osteochondral fragments

and cartilage explants could have a variety of changes in matrix content (GAG and COL) depending on the culture conditions [7, 9, 27, 28, 32, 49, 54, 55], whereas 37°C samples containing viable chondrocytes may be able to maintain cartilage homeostasis and prevent tissue degeneration following implantation. The results in this study reported that GAG and COL could be maintained for osteochondral samples stored at 37°C for 28 days. While matrix content measurements are not as sensitive to changes as synthesis and degradation rates, they can typically be correlated with mechanical properties of the tissue.

The results of the present study suggest that osteochondral grafts stored at 37°C for 2 to 4 weeks have better biological performance than grafts stored at 4°C for the same duration. Cell death in the superficial zone may have deleterious effects after implantation, as chondrocytes in this zone normally secrete a lubricant molecule proteoglycan-4 (PRG4) [51]. PRG4 has a boundary-lubricating ability [50], and has been shown to protect the cartilage surface from wear by reducing friction when compared to PRG4 deficient joints [31]. During storage, reduction of viable chondrocytes in the superficial zone and their secreted lubricants may be key factors in graft failure. Studies to investigate more closely the relationship between chondrocyte viability and function through the production of zonal-specific molecules may provide additional insight into the factors necessary for graft success. Storage alternatives, including physiological temperature, could increase the storage duration window for suitable donor tissue and provide higher quality graft tissue.

The present study also suggests that storage at 37°C, compared to traditional 4°C, may allow extended storage duration of grafts, during which chondrocyte viability and cartilage matrix content are maintained. 37°C storage maintained chondrocyte viability, especially in the superficial zone, although the mechanism of

cell death and its variation within the zones of cartilage are still topics of speculation. Chondrocytes at the surface may be more susceptible to storage-associated death because their native physical and chemical environment is disrupted, whereas chondrocytes in the deeper regions of cartilage are in a matrix-filled environment that more closely resembles the native form. Preserving chondrocyte viability during prolonged storage intervals could facilitate increased graft shelf-life and availability of osteochondral allografts. However, grafts stored at 37°C rather than 4°C may have a higher incidence of infection and spoilage because microorganisms typically grow faster at such elevated temperatures. In addition, conditions that maintain viability in the superficial zone, in particular, may improve clinical performance by providing lubricating molecules, such as PRG4, to maintain a low-friction articulating surface. The relationship among overall chondrocyte viability, superficial zone viability, and subsequent success after osteochondral allografting remains to be elucidated.

6.6 Acknowledgments

Table 6.1: Storage conditions

Storage Condition	Temperature [°C]	FBS [%]	Duration [days]
i	FRESH CONTROL		0
ii	4	10	14
iii	4	10	28
iv	37	0	28
v	37	2	28
vi	37	10	28

Table 6.2: Cartilage thickness, GAG and COL content of fresh and stored goat osteochondral samples. Data are mean ± SEM.

Storage Condition	n	Thickness [mm]	n	GAG [mg/g]	COL [mg/g]
i	18	0.71 ± 0.20	9	34.7 ± 3.1	117.6 ± 8.4
ii	13	0.71 ± 0.20	4	41.7 ± 8.6	118.2 ± 8.0
iii	10	0.68 ± 0.19	4	40.9 ± 14.1	101.6 ± 7.5
iv	7	0.83 ± 0.13	7	27.4 ± 3.7	120.8 ± 8.2
v	7	0.85 ± 0.09	7	27.9 ± 3.8	114.8 ± 5.2
vi	10	0.68 ± 0.24	4	23.2 ± 5.5	121.5 ± 9.8
p-values		0.2		0.2	0.7
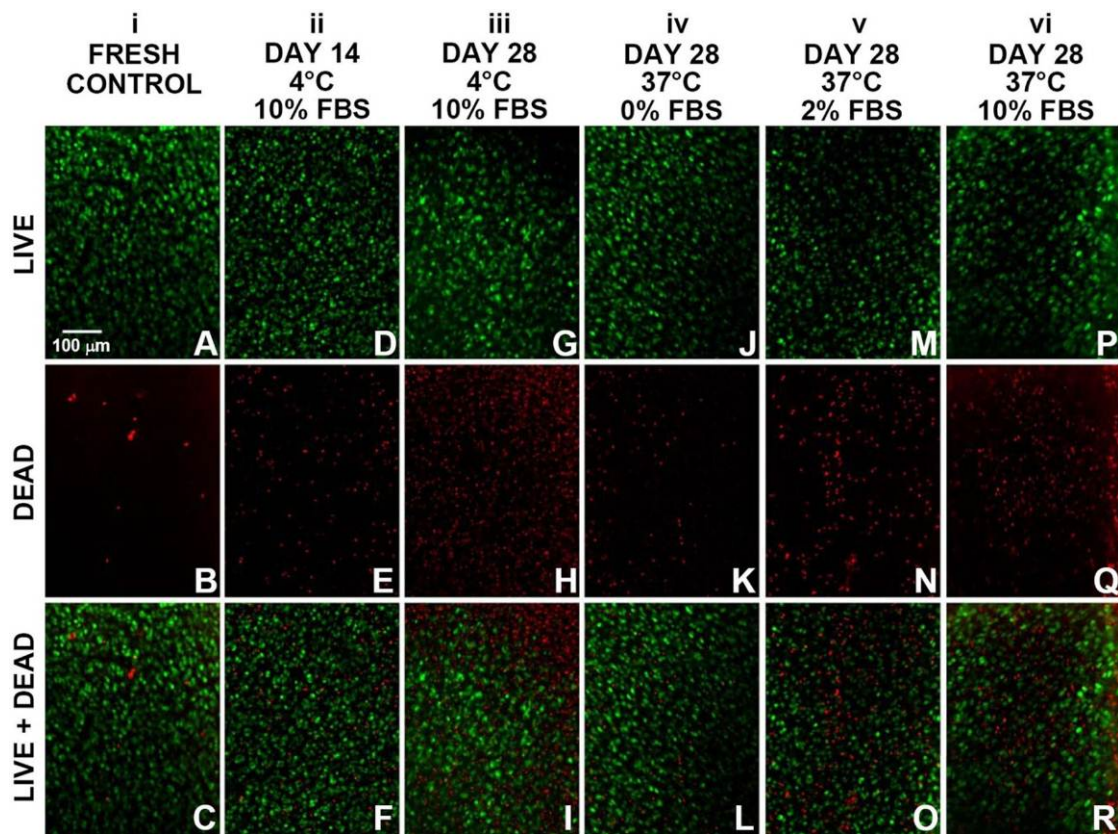
Figure 6.1: *En face* view of chondrocyte viability at the articular surface in goat osteochondral samples, as analyzed by LIVE/DEAD® fluorescence staining. (**A-C**) Fresh controls. Samples stored at 4°C in medium including 10% FBS for (**D-F**) 14 days and (**G-I**) 28 days, as well samples stored at 37°C for 28 days in medium including (**J-L**) 0% FBS, (**M-O**) 2% FBS, or (**P-R**) 10% FBS. (**A,D,G,J,M,P**) Images of live cells (green fluorescence). (**B,E,H,K,N,Q**) Images of dead cells (red fluorescence). (**C,F,I,L,O,R**): Merged live+dead images.
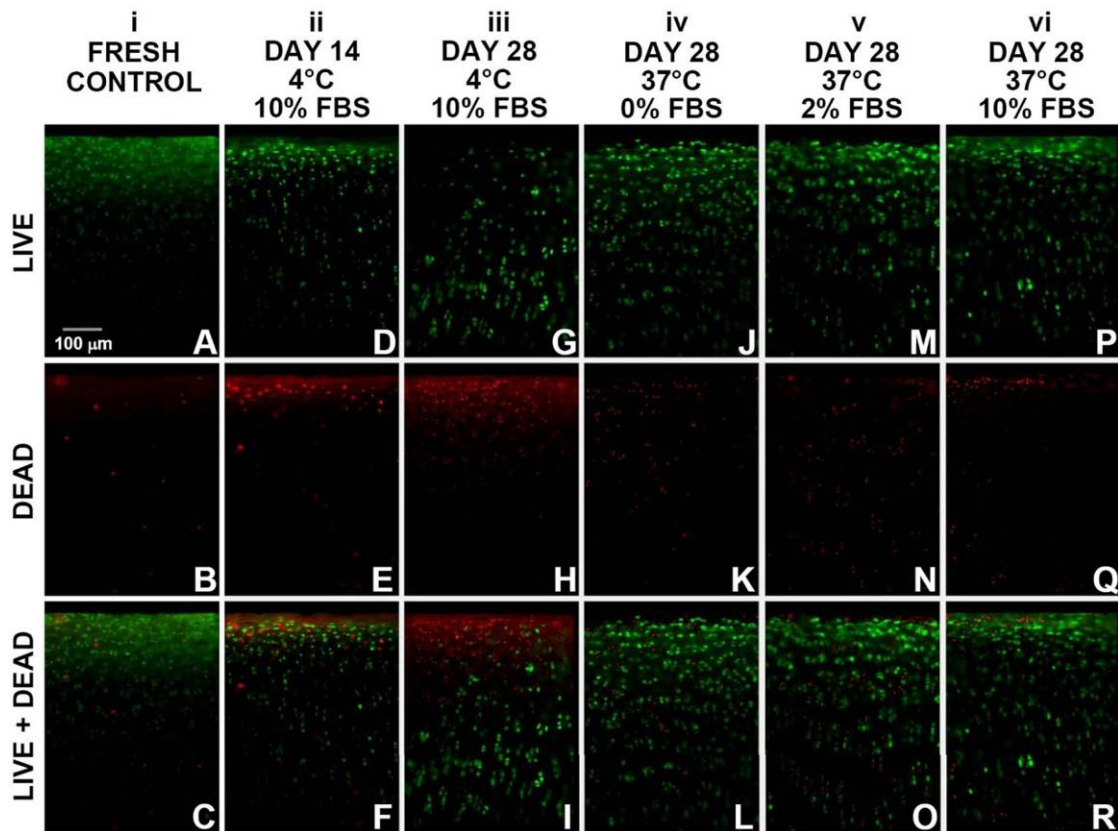
Figure 6.2: Vertical profile view of chondrocyte viability throughout the depth of articular cartilage in goat osteochondral samples, as analyzed by LIVE/DEAD® fluorescence staining. (**A-C**) Fresh controls. Samples stored at 4°C in medium including 10% FBS for (**D-F**) 14 days and (**G-I**) 28 days, as well samples stored at 37°C for 28 days in medium including (**J-L**) 0% FBS, (M-O) 2% FBS, or (**P-R**) 10% FBS. (**A,D,G,J,M,P**) Images of live cells (green fluorescence). (**B,E,H,K,N,Q**) Images of dead cells (red fluorescence). (**C,F,I,L,O,R**): Merged live+dead images.
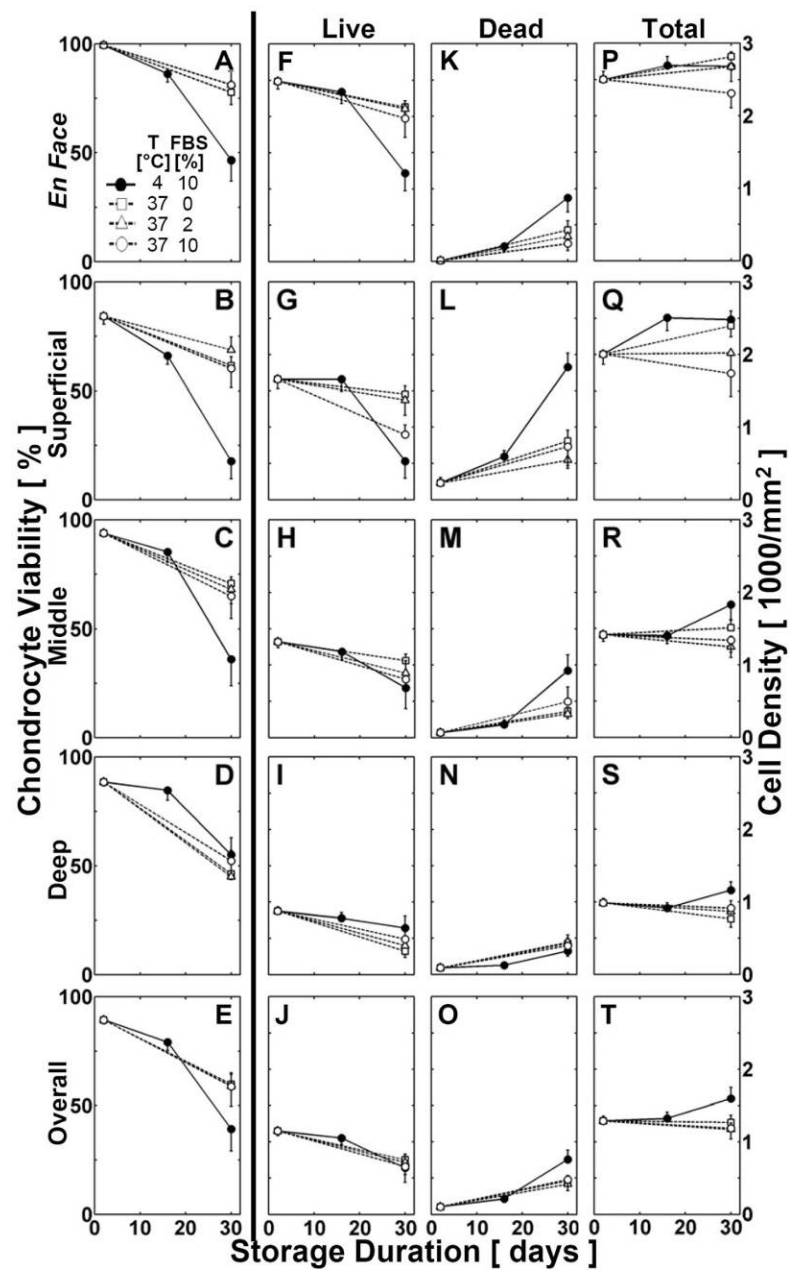
Figure 6.3: Effects of storage condition on chondrocyte viability, live, dead, and total cell density throughout the depth of cartilage from osteochondral samples over a storage duration of 28 days. Chondrocyte viability and cell densities at (**A,F,K,P**) the cartilage surface *en face*, by region for the (**B,G,L,Q**) superficial [15%], (**C,H,M,R**) middle [35%], and (**D,I,N,S**) deep [50%] zones, and (**E,J,O,T**) in the overall vertical profile.

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CHAPTER 7:

BONE CYSTS AFTER OSTEOCHONDRAL ALLOGRAFT REPAIR OF CARTILAGE DEFECTS IN GOATS SUGGESTS ABNORMAL INTERACTION BETWEEN SUBCHONDRAL BONE AND OVERLYING SYNOVIAL JOINT TISSUES

7.1   Abstract

The efficacy of osteochondral allografts (OCA) may be affected by osseous support of overlying articular cartilage, and thus affected by bone integration and remodeling in the OCA and surrounding host. Bone cysts, and their communication pathways, may be present in various locations after OCA insertion and reflect distinct pathogenic mechanisms. Previously, we analyzed the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on cartilage quality and biology in fifteen goats after 12 months *in vivo*. The objectives of the present study were to further analyze OCA and contralateral CONTROL samples from the medial femoral condyle to (a) determine the effect of OCA storage on local subchondral (ScB) and trabecular (TB) bone structure, (b) characterize the structure of bone cysts, and (c) assess the relationship between cartilage and bone properties. Bone structure was altered by OCA relative to contralateral non-operated (Non-Op) control knees. Structural μCT bone scores and bone architecture, for both ScB and TB, was similar among FRESH, 4°C/14d, and

4°C/28d OCA; however, FROZEN OCA had worse structural μCT bone scores and increased ScB erosion versus other OCA. TB in peri-cyst wall regions of OCA exhibited higher BV/TV than Non-Op. OCA repair patterns all exhibited basal cysts, and occurred (a) in isolation, (b) with surface cysts and surface channels, (c) with surface channels, or (d) with surface cysts, surface channels, and ScB erosion. Deterioration of cartilage gross morphology was strongly associated with decreased μCT bone structure. These findings suggest that bone cysts occurring after OCA may result from aberrant mechanobiology due to (1) bone resorption at the graft-host interface, (2) abnormal channels between synovial fluid and underlying bone, and/or (3) deterioration of the articular cartilage or ScB tissues. The latter two processes involve abnormal interactions between the subchondral bone and overlying synovial joint, which are normally compartmentalized.

7.2   Introduction

The efficacy of osteochondral allografts (OCA) for repairing defects of articular cartilage may be affected by subchondral bone integration, as determined by remodeling of both the allograft and surrounding host bone. Clinically, graft failure is often associated with boney non-union, late fragmentation, and collapse due, all indicative of insufficient osseous support; such failure is often accompanied by persistent bone sclerosis, bone resorption, and subchondral bone cysts, the latter being identified as well-demarcated and often rounded areas of translucency in bone [6, 13, 16, 23, 35, 39]. Subchondral bone cysts also result in animal models following osteochondral allografting [1, 27, 29, 38], and other cartilage repair treatments [4, 5, 12, 25]. Articular cartilage often deteriorates (decreased volume, histopathological indices of degeneration, decreased stiffness) near bone lesions [3, 8, 21, 30, 37]; However, the etiology of such subchondral bone pathology and its relationship to overlying articular cartilage is unclear, particularly in the setting of OCA.

In OCA repair, subchondral bone cysts may arise from mechanisms analogous to those proposed for the formation of such cysts after injury and in osteoarthritis. The *Synovial Fluid Intrusion* hypothesis suggests that subchondral bone cysts form from degenerative changes of the overlying cartilage, leading to aberrant communications between the joint space and subchondral bone and forcing pressurized synovial fluid exudation into the subchondral bone; such fluid intrusion alters mechanical and biological homeostasis in the bone [7, 17]. This mechanism is plausible in the setting of OCA due to the perturbation of the articular cartilage layer by the implant. Alternatively, the *Bony Contusion* hypothesis suggests that subchondral bone cysts form as a biological reaction of the subchondral bone in response to excessive, concentrated loads on the bone; such bony contusion leads to decoupled bone

metabolism favoring resorption over formation [24]. This mechanism may be relevant to OCA, either through excessive loading of bone, during or after OCA insertion; alternatively, an abnormal bone metabolism may be due to unloading of bone, due to gaps between implant and host. The structural properties and relationships between cartilage and underlying bone, including cysts, following OCA repair may not only implicate the etiology of bone cysts, but also suggest approaches to enhance cartilage repair.

Some methods have been established for assessing the structure of subchondral bone cysts and surrounding bone microarchitecture, mapping nearby bone channels and regions of subchondral bone deterioration. Cysts are well-demarcated as substantial regions, devoid of trabecular bone and on $\mu(CT)$/histology [25], and with signal intensity similar to that of joint cavity fluid on MRI [3]. Cyst size is typically measured in the image slice with the greatest lesion size, semi-quantitatively [15, 19, 25, 28, 37], quantitatively as the maximum diameter in a selected plane [21], or as volume estimated from three orthogonal slices [3]. Subchondral bone discontinuities, allowing direct communication between the joint space and trabecular bone have been identified [14, 17, 21, 31, 32]; however, the extent of such discontinuities and their relationship to bone cysts have not been examined. Bone microarchitecture around cysts is sometimes dense at the walls, then more porous and branched with a relatively normal overall bone volume fraction in surrounding regions, and accompanied by increased porosity of the subchondral bone [5, 22]; methods for regional bone evaluation have not yet been applied to bone cysts [20]. Future improvements in the application of OCA would benefit from an integrative understanding of the structural relationship among cartilage, bone, and cysts following repair.

Thus, the objectives of the study were to (a) determine the effect of OCA

storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on subchondral and trabecular bone structure in the graft region, (b) characterize the structure of bone cysts, and (c) assess the relationship between cartilage and bone properties following OCA repair at 12 months in the goat. The results of the analysis suggest mechanisms contributing to the development of bone cysts following cartilage defect repair by osteochondral allografts.

7.3   Materials and Methods

OCA Samples

The effects on bone structure of cartilage defect repair with stored OCA after 12 months *in vivo* were analzyed using tissue from a previous study in an adult goat model, with IACUC approval [26]. Osteochondral cores (d=15mm, h~8-10mm), which encompassed the experimental graft site and portions of the surrounding host tissue, from FRESH, 4°C/14d, 4°C/28d, and FROZEN OCA (each, n=3-4) at the medial femoral condyle (MFC), were retrieved after sacrifice and analyzed for bone structure by micro-computed tomography (μCT) and histology with hematoxylin and eosin (H&E) staining. Site-matched regions on contralateral knees were Non-Operated (Non-Op) controls (n=15), and were analyzed analogously. Relationships between cartilage properties reported previously [26], including cartilage gross morphology, superficial zone cellularity, matrix fixed charge, and stiffness, and bone properties were assessed by principal component analysis.

Micro-Computed Tomography (μCT)

μCT imaging was performed for 3D visualization and morphometric quantification of bone architecture and structure in the retrieved OCA and site-matched Non-Op samples. Osteochondral samples were submerged in tissue culture medium (low-glucose Dulbecco's modified Eagle's medium) and imaged in 3D using μCT (Skyscan 1076, Kontich, Belgium): 70kV, voxel=$(18\mu m)^3$. Sample volumes were oriented using DataViewer software (version 1.4.3; Skyscan) such that transverse axis was parallel to the proximal and distal interface between the graft and host bone in both the sagittal and coronal imaging planes (Fig. 7.1A), cropped to a $(580px)^3$ region, and saved as a new data set. Quantitative 3D morphometric bone analysis was

performed using CTAnalyser software (version 1.11.10.0; Skyscan), and reported using standard nomenclature[2].

Bone Structure Analysis of OCA Graft Region

The bone structure in the OCA, as visualized by μCT, was evaluated semi-quantitatively for bone cyst size, bone channel size, and bone-cartilage interface roughness using 3D μCT data. The resulting bone structure score was on a 0-9 scale, with high scores corresponding to deterioration and/or poor repair. Cyst size was scored based on cumulative cyst volume (V) for the entire osteochondral sample, as no cysts (score=0), $V<5mm^3$ (score=1), $V=5-15mm^3$ (score=2), or $V>15mm^3$ (score=3). Bone channel size was scored across the graft and host bone for the degree of disconnect across the subchondral bone plate, as intact (score=0), containing one small gap (<0.5mm, score=1), one large gap (0.5-2.0mm, score=2), or extra-large/multiple gaps (cumulative gap>2.0mm, score=3). Bone-cartilage interface roughness was scored as smooth (score=0), containing either a step-off >0.5mm at the graft-host interface or mild roughening (score=1), containing both a step-off >0.5mm at the graft-host interface and mild roughening (score=2), or as severe roughening with or without a step-off (score=3). Scores were averaged from three blinded observers.

Histological structure in the OCA was also assessed by evaluation of the bone graft-host integration, calcified cartilage, and bone marrow in sections stained with H&E. Histological sections were scored through a central proximal to distal path within a $5.0x2.5mm^2$ region (Fig. 7.1D). Calcified cartilage was assessed as intact (+) or as thinning/absent (—); the presence of multiple tidemarks were also noted. Bone marrow was scored from normal (with typical presence of fat cells, score=0) to >66%

fibrosis (score=3). Bone cysts, when visible, were also examined for edema (characterized by eosinophilic extracellular fluid), fibrosis (characterized by collagenous fibers and spindle-shaped purple fibroblasts), and trabecular abnormalities (including necrosis, sclerosis, and remodeling), which are common histologic features associated with bone marrow lesions [40].

Subchondral bone plate (ScB) architecture was assessed at the bone surface in the central graft region by μCT (Fig. 7.1B). Graft ScB volume of interest (VOI), from the bone surface to a depth of 0.54mm within the central graft region (d=5mm), was segmented from the sagittal plane in Matlab (Mathworks, Natick, MA) by identifying the bone surface contours using thresholds,(10,11) and imported into CTAnalyzer. ScB within this VOI was segmented using a global threshold (125-255), and analyzed for bone volume fraction (BV/TV, %) and specific bone surface (BS/BV, mm^{-1}).

Trabecular bone architecture was assessed from a cylindrical VOI of bulk graft bone centered below the bone surface by μCT (Fig. 7.1C). The transverse slice that corresponded to the proximal interface between the graft and host bone ($Z_{HOST, Proximal}$) was identified in DataViewer. Graft trabecular bone VOI was defined 0.36mm from $Z_{HOST, Proximal}$, 2.4mm deep (from $Z_{TRAB, Surface}$ to $Z_{TRAB, Deep}$) within the central graft region (d=6mm). Trabecular bone within this VOI was segmented adaptively with the Otsu method using CTAnalyzer software, and analyzed for bone volume fraction (BV/TV, %), bone surface density (BS/TV, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular spacing (Tb.Sp, mm), and trabecular number (Tb.N, mm^{-1}).

Structural Bone Cyst Analysis

Bone cysts in OCA were localized in 3D as large void regions within μCT data sets (Fig. 7.1E). Data sets were thresholded and processed in 3D to close trabecular

gaps and isolate the void regions in Matlab. Isosurface data was extracted from the 3D void volume, and fit to an ellipsoid using least squares ellipsoid specific fitting [18] to estimate cyst volume. Osteochondral samples from Non-Op did not contain bone cysts, and were not analyzed for cyst volume.

From μCT 3D data sets, bone cysts were characterized into groups based on location, presence of a bone surface channel and its relationship to a surrounding cyst, and subchondral bone integrity within the OCA. Bone cysts that were localized to deeper regions of the sample were classified as "basal" cysts, while bone cysts that were localized near the bone-cartilage interface were classified as "surface" cysts. Bone surface channels were identified as visible gaps (ranging in width from 0.2-3mm) at the bone-cartilage interface, and 3D renders of the bone-cartilage interface were created in Mimics (Materialise, Belgium) to localize these channels. Bone surface channels were then examined for connections to surface cysts. Subchondral erosoin was assessed visually from μCT data sets versus corresponding Non-Op bone, and confirmed with interpretation from ScB BV/TV measurements.

Bone architecture in the peri-(basal) cyst wall was assessed in a 1mm thick shell surrounding the localized 3D bone cyst region by μCT (Fig. 1F). Although Non-Op samples did not contain cysts, ellipsoids (fit to the basal cyst of the corresponding OP joint) were site-matched in Non-Op samples (relative to $Z_{HOST, Proximal}$) as pseudo-cyst regions, and bone structure was analyzed in the 1mm thick wall surrounding the ellipsoid. Bone volume fraction (BV/TV, %), bone surface density (BS/TV, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular spacing (Tb.Sp, mm), and trabecular number (Tb.N, mm^{-1}) were then calculated within the peri-cyst wall VOI. For some Non-Op samples, pseudo-cyst regions were placed at different locations (up to 5mm medial, lateral, and distal to original location) and bone architecture parameters were

within 5% at each location.

Statistics

Data are presented as mean±SEM, with p<0.05 considered significant. Statistical analysis was performed as a paired design on the difference between matched OP and Non-Op samples. For non-parametric data (i.e. structural bone scores), the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on overall bone score, and the individual components (bone cyst severity, bone channel size, and subchondral bone roughness) were determined by Kruskal-Wallis and Dunn's *post hoc* tests. For quantitative μCT data, the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on subchondral (ScB BV/TV, ScB BS/BV) and trabecular (BV/TV, BS/TV, Tb.Th, Tb.Sp, Tb.N) bone structure in the graft and peri-cyst wall (trabecular structure only) regions were determined by ANOVA with Tukey *post hoc* tests. In addition, planned comparisons were performed between each OCA storage group and Non-Op (i.e. population=0) with a one-sample *t*-test. Statistical analyses were performed using Systat 10.2 (Systat Software Inc., Chicago, IL).

In addition, to determine which of the many cartilage and bone parameters were closely related to each other, a factor analysis by the method of principal components was performed. Cartilage properties from stored OCA were reported previously as measures of cartilage integrity [26]. Cartilage properties, including gross score, superficial cellularity, hexabrix gray value (inverse of matrix fixed charge), and material stiffness, OCA graft bone properties, including structural score, subchondral architecture, and trabecular architecture, and peri-cyst wall bone architecture were the parameters included in this analysis. The principal components matrix was computed, and the loading coefficients (describing how each parameter contributed to each

factor) were obtained. For each factor, the dominant contributors were identified as those whose coefficients (ranging from -1 to +1) had an absolute value greater than or equal to 0.33.

7.4   Results

Structural μCT bone scores (except bone cyst size) varied with OCA storage (each, $p<0.01$, Table 7.1). Bone structure after OCA was altered compared to Non-Op joints, with OCA samples displaying bone cysts, and bone surface channels with or without roughening at the bone-cartilage interface, and all Non-Op joints scoring 0. Overall structural bone score in Non-Op was lower than FRESH ($p<0.01$), 4°C/14d ($p<0.05$), 4°C/28d ($p<0.01$), and FROZEN ($p<0.001$) OCA storage treatments (Table 1, Fig. 7.2A-E). Overall structural bone scores in 4°C/14d was similar to 4°C/28d ($p>0.6$) and FRESH ($p>0.2$), and lower than FROZEN ($p<0.01$). Bone cyst size was similar among OCA treatments ($p>0.3$), and much worse than Non-Op (each, $p<0.01$) as no Non-Op joints contained bone cysts. Bone surface channel size in 4°C/14d, which was small (<0.5mm), tended to be larger than Non-Op ($p=0.07$) and 4°C/28d ($p=0.09$), but was smaller than FRESH ($p<0.05$) and FROZEN ($p<0.01$) OCA, in which channel sizes were larger (>0.5mm) (Fig. 7.S1). The bone-cartilage interface was smooth in Non-Op, slightly rougher in FRESH, 4°C/14d, and 4°C/28d ($p>0.05$), and rougher in FROZEN ($p<0.001$ vs. Non-Op, $p<0.05$ vs. other OCA, Fig. 7.S1).

Histologically, all OCA exhibited bony union at the circumferential graft-host junction, but were variably accompanied by histopathological structural abnormalities within the bone of OCA (Fig. 7.2F-T). The calcified cartilage was typically intact in Non-Op joints (15/15), as well as FRESH OCA (3/3), but absent in all FROZEN (4/4) and some 4°C stored OCA (3/4 for 4°C/14d, 2/4 for 4°C/28d, Fig. 7.2K-O); although some Non-Op and FRESH OCA had multiple tidemarks (8/15 for Non-Op, 1/3 for FRESH). Bone marrow in Non-Op exhibited typical presence of fat cells (11/15) or minimal fibrosis (i.e. <33%, 4/15); that minimal fibrosis was seen in most FRESH (3/3), 4°C/14d (4/4), and 4°C/28d (3/4) OCA, but predominant fibrosis was evident in

FROZEN (3/4) OCA (Fig 7.2P-T).

Subchondral bone plate µCT architecture in the graft region, normalized to contralateral Non-Op controls, varied with OCA storage (each, $p<0.001$). ScB architecture was altered in Non-Op versus FROZEN OCA, but not versus all other OCA. ScB bone volume fraction (BV/TV) and ScB bone surface density (BV/BS) in Non-Op was $90\pm2\%$ and 7.5 ± 0.7mm^{-1}, respectively, and was similar to FRESH ($p=1.0$), 4°C/14d ($p>0.7$), and 4°C/28d ($p<0.6$) OCA (Fig. 7.3). ScB BV/TV in FROZEN was $49\pm2\%$, and was ~30-40% lower than Non-Op, FRESH, 4°C/14d, and 4°C/28d OCA (each, $p<0.001$, Fig. 3A). Concomitantly, ScB BS/TV in FROZEN was 20.9 ± 1.4mm^{-1}, and ~2-3x times higher than Non-Op, FRESH, 4°C/14d, and 4°C/28d OCA (each, $p<0.001$, Fig. 7.3B).

In the trabecular bone region of OCA, some trabecular bone µCT architecture parameters, normalized to contralateral Non-Op controls, varied with OCA storage (BS/TV and Tb.N, each, $p<0.05$), while others did not (BV/TV, Tb.Th, and Tb.N, each $p>0.3$). In general, trabecular bone structure was similar among FRESH, 4°C/14d, and 4°C/28d OCA, with differences in bone architecture parameters versus both Non-Op controls and FROZEN OCA, but no difference in average bone volume density. Bone volume fraction (BV/TV) was similar among OCA storage, and similar to Non-Op ($p>0.7$, Table 7.2). Bone surface density (BS/TV) and trabecular number (Tb.N) in Non-Op was similar to FROZEN ($p>0.6$), and higher than FRESH, 4°C/14d, and 4°C/28d (each, $p<0.05$, Table 7.2). Trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp) was similar among OCA storage ($p>0.3$); however, Tb.Th and Tb.Sp were higher in 4°C/14d and 4°C/28d OCA compared to Non-Op (each, $p<0.05$, Table 7.2).

Subchondral bone cysts were widespread after defect repair by OCA at 12

months, with variable cyst volume and location. Twenty-two bone cysts (volume=8.5±1.3mm^3, range=0.8-23.3mm^3), were identified by µCT in 15 OP joints (Fig. 7.4). All OP joints (15/15) contained a bone cyst that was located in the deeper region of the graft, which was classified as a "basal" cyst (15 basal cysts). In addition, some OP joints (6/15) contained bone cysts that were near the bone-cartilage interface, which were classified as "surface" cysts (7 surface cysts). Basal cysts were typically larger in volume than surface cysts (11.2±1.5mm^3 vs. 2.7±0.6mm^3, respectively). Some OP joints had bone surface channels (10/15 joints contained 11 bone channels), which were identified as breaks in the bone-cartilage interface, and ranged in size from 0.4-3mm wide (Fig. 7.S1). Some of these bone channels (6/11) connected the nearby surface cyst volume to the overlying cartilage, while other channels (5/11) penetrated into the trabecular bone, but did not directly connect to a cyst.

Histologically, the structure of and contents within bone cysts appeared to vary with the location of the cyst. Non-Op control bone did not display histological evidence of bone cysts. Some surface cysts, which were associated with a bone surface channel, contained fibrous and collagenous tissue (Fig. 7.S2A-D). Other basal cysts contained normal bone marrow, displaying primarily fat cells (Fig. 7.S2E-H); not all cysts could be evaluated histologically due to the sampling through the central proximal to distal path. Other histologic features typically associated with bone marrow lesions were displayed, including swollen fat cells and bone marrow edema (Fig. 7.S2B), and abnormal trabecular remodeling, including necrosis (Fig. 7.S2E), reversal lines (Fig. 7.S2E), and bone sclerosis (Fig. 7.S2H).

Peri-(basal)cyst wall µCT architecture was different than Non-Op bone, but did not vary with OCA storage (p>0.2). Peri-(basal)cyst wall architecture in OCA generally had higher BV/TV, BS/TV, and Tb.N, lower Tb.Sp, and similar Tb.Th

compared to Non-Op (Table 7.3). However, peri-(basal)cyst wall BV/TV in OCA was variable and ranged from 0-15% higher than Non-Op. Peri-(basal)cyst wall BV/TV was similar in some OCA (7/15; each, $\Delta\leq5\%$), and much higher in other OCA (8/15; each, $\Delta\geq10\%$) compared to Non-Op. In OCA with increased peri-(basal)cyst wall BV/TV, Tb.Th was ~30% higher and Tb.Sp was ~15% lower than Non-Op samples.

Considering all OP joints, retrieved OCA contained a mix of different bone cyst and channel types; four patterns of cyst, channel, and subchondral bone erosion mixes were identified (Fig. 7.5). Some OCA contained basal cysts only (Group A, Fig. 7.5A); the peri-(basal)cyst wall architecture was similar to Non-Op. Some OCA contained both basal and surface cysts, with bone surface channels (Group B, Fig. 7.5B); the peri-(basal)cyst wall bone architecture had higher BV/TV, BS/TV, and Tb.N, and lower Tb.Sp compared to Non-Op. Some OCA contained basal cysts and bone surface channels, but without surface cysts (Group C, Fig. 7.5C), in which the peri-(basal)cyst architecture tended to have higher BV/TV and Tb.Th compared to Non-Op. Other OCA with both basal and surface cysts and bone surface channels were also accompanied by subchondral bone erosion (Group D, Fig. 7.5D); the peri-(basal)cyst wall architecture was similar to that in Group B. When the subchondral bone surface was intact, bone surface channels were typically at the periphery of the OCA near the graft-host interface (Fig. 7.5B,C); bone surface channels were typically at the center of the OCA when the subchondral bone surface was eroded (Fig. 7.5D,E).

Principle component analysis demonstrated that gross morphology of the cartilage surface and μCT bone structure were strongly and positively related (Table 7.4). In addition, subchondral bone integrity (ScB BV/TV and ScB BS/BV) was related to bone volume fraction of the underlying trabecular bone and the bone surrounding basal cysts.

7.5   Discussion

These results demonstrate that altered bone structure in OCA versus Non-Op bone was similar among FRESH, 4°C/14d, and 4°C/28d OCA, and worse in FROZEN OCA, and suggest that OCA repair patterns are related to mechanisms of bone failure in the setting of OCA. FRESH, 4°C/14d, and 4°C/28d OCA displayed graft bone with an intact subchondral bone plate and minimal marrow fibrosis similar to Non-Op, but altered architectural and histopathological abnormalities, including bone cysts (Figs. 7.2-4, Tables 7.1-3). In contrast, FROZEN OCA exhibited eroded subchondral bone plate and predominantly marrow fibrosis, also accompanied by cysts, but trabecular architecture that was similar to Non-Op (Figs. 7.2-4, Tables 7.1-3). The association of cartilage gross morphology and overall μCT bone structure suggests that subchondral bone pathology is related to the overlying articular cartilage integrity (Table 7.4). Characterization of OCA based on cyst location, bone surface channel size, and subchondral bone integrity identified patterns of altered bone architecture in the peri-(basal)cyst wall (Fig. 7.5), associations of structural and histological abnormalities, and variations of bone failure within OCA storage groups, which may help to elucidate factors that are critical to the prevention of bone cysts following cartilage repair.

Examining bone and cyst structure after cartilage restoration by osteochondral allografting involved the consideration of a number of issues. Since samples retrieved 12months after OCA were used, the ability to directly observe an integrative view of bone and cartilage structure and biology was counter-balanced by an inability to analyze the time progression of lesions in individual samples. It would be interesting to determine if progression pathways can be predicted from cross-sectional data. Despite the variability between animals and limited sample numbers for each storage

condition, the 15 operated knees represented a reasonable spectrum of bone lesions at different stages of progression, and there were still clear differences in overall bone structure and subchondral bone integrity between storage conditions.

Subchondral bone plate deterioration in FROZEN allografts, in association with degradation of the articular cartilage [26], suggests that cartilage and bone coupling may affect biological and structural remodeling during osteochondral repair. Such FROZEN OCA exhibited severe cartilage degeneration at 12months with softening, loss of chondrocytes, reduced proteoglycan content, and visible structural deterioration of the articular surface and bulk tissue, likely indicating cartilage failure [26]. The inability of cartilage in FROZEN OCA to maintain fluid pressurization and sustain joint loads may result in transmission of excessive loads and exudation of pressurized fluid into the underlying bone, altering the typical mechanical and biological bone environment (Figs. 7.2,3,5, Tables 7.1-3). The strong association between cartilage and bone structure (Table 7.4) suggests that cartilage failure may lead to subsequent bone failure.

Regional bone evaluation of peri-(basal)cyst wall architecture allowed for quantification of structural abnormalities in allograft bone near visible bone cysts. Using standard geometric μCT VOIs, measures of trabecular architecture in samples with visible bone cysts, especially bone volume fraction, are often unaffected, as voids and dense regions balance out, and cysts are simply identified qualitatively or scored semi-quantitatively [21, 22, 25]. Standardized methods to create VOIs that account for joint curvature have been used to more precisely analyze bone architecture [20], and have identified that bone structure is more porous and branched in regions containing bone cysts [5]; however, the architecture directly surrounding the visible bone lesions has not been examined. In this study, the peri-(basal)cyst wall was similar to non-

operated bone in some samples, and denser, as evidenced by increased bone volume fraction, in other samples (Fig. 7.5). The variability in cyst wall architecture may distinguish two distinct pathologies of base bone cysts, which may require unique treatments specific to each of the cyst pathologies.

The association of structural and histological abnormalities in bone healing within allografts suggests that altered biological bone homeostasis contributes to the observed structural pathologies. Subchondral bone plate deterioration, especially in frozen allografts, and the presence of bone surface channels were associated with increased fibrous marrow in the underlying bone. The contents of surface cysts, often connected to the cartilage by bone surface channels, were accompanied by edema and fibrous marrow, while basal cysts contained primarily fat cells surrounded by necrotic and sclerotic bone (Fig. 7.S2). In addition, bone healing may be influenced, in part, by the immunogenicity of allogenic bone, as reducing surface antigens (by freezing) [10, 33] or matching leukocyte-antigens, has improved osseous integration and clinical outcomes [11, 36]. While antigen matching was not attempted in this study, in accordance with current clinical practice, it may be a confounding factor. Thus, future studies investigating bone metabolism and its relationship to cyst structure during cyst development may further elucidate potential biological mechanisms of cyst formation.

Characterization of OCA based on cyst structure may help to identify potential biophysical and biological mechanisms of bone cyst formation following cartilage repair (Fig. 7.6). Simple basal cysts may result from in adequate bone remodeling (Fig. 7.6A); new bone formation may not be able to fill voids created during surgery due to issues with graft fit and surgical tools (i.e. guidewires), but overall bone and cartilage structure is relatively unaffected. Bone surface lesions may induce surface cysts (Fig. 7.6B), introducing pressurized fluid into the bone and leading to a

resorptive cavity near the surface; overall cartilage structure is relatively unaffected, but bone marrow fibrosis and peri-(basal)cyst wall thickening results. Bone cysts may also result from cartilage depressurization through either bone surface channels, typically at the peripheral graft-host interface, (Fig. 7.6C) and/or cartilage degeneration (Fig. 7.6C,D) and increased subchondral bone permeability; "leaky" cartilage alters both biological and mechanical homeostasis in the osteochondral unit resulting in subchondral bone degeneration and altered peri-(basal)cyst bone architecture. While specific pathological states of bone cysts after osteochondral allografting are identified here and may be useful to identify potential interventions tailored to each state, the time progression of such lesions remains to be elucidated.

7.6   Acknowledgments

Table 7.1: Effect of OCA storage on bone structure after 12months *in vivo*. [c]Structural bone score (0-9) was determined by assigning a score of 0-3 (with 0 representing normal, and 3 representing severe degeneration) for the following bone characteristics: bone cyst severity, bone channel size, and bone-cartilage interface roughness.

Mean±SEM. *p<0.05, **p<0.05, ***p<0.001 vs. Non-Op. Letters (a and b) designate significantly different groups (p<0.05), with a shared letter indicating no difference.

	Non-Op	FRESH	4°C/14d	4°C/28d	FROZEN
n	15	3	4	4	4
Structural Bone Score[c]	0	6.1±0.4** ab	3.6±0.6* a	4.8±0.6** ab	7.3±0.9*** b
Bone Cyst Severity	0	2.7±0.3** a	2.0±0.3** a	1.8±0.5** a	2.3±0.5** a
Bone Channel Size	0	2.3±0.3** a	0.4±0.3 b	1.8±0.5* ab	2.4±0.5** a
Interface Roughness	0	1.1±0.1* a	1.1±0.5* a	1.2±0.4* a	3.0±0.0*** b

Table 7.2: Effect of OCA storage on graft trabecular bone architecture after 12months *in vivo*. Mean±SEM. *p<0.05 vs. Non-Op. Letters (a and b) designate significantly different groups (p<0.05), with a shared letter indicating no difference.

		Difference = OP – Non-Op			
	Non-Op	**FRESH**	**4°C/14d**	**4°C/28d**	**FROZEN**
n	15	3	4	4	4
BV/TV [%]	53±2	0±4 a	2±1 a	−2±2 a	1±4 a
BS/TV [mm^{-1}]	6.5±0.1	−0.7±0.2* ab	−0.9±0.2* a	−1.0±0.2* a	0.1±0.4 b
Tb.Th [mm]	0.25±0.01	0.03±0.02 a	0.05±0.01* a	0.04±0.01* a	0.01±0.02 a
Tb. Sp [mm]	0.35±0.01	0.06±0.04 a	0.04±0.01* a	0.09±0.05* a	0.02±0.02 a
Tb.N [mm^{-1}]	2.17±0.05	−0.25±0.05* a	−0.33±0.08* a	−0.35±0.09* a	−0.06±0.12 b

Table 7.3: Effect of OCA storage on peri-(basal)cyst wall bone architecture after 12months *in vivo*. Mean±SEM. *p<0.05 vs. Non-Op. Letters (a and b) designate significantly different groups (p<0.05), with a shared letter indicating no difference.

		Difference = OP – Non-Op			
	Non-Op	**FRESH**	**4°C/14d**	**4°C/28d**	**FROZEN**
n	15	3	4	4	4
BV/TV [%]	39±2	10±1*** a	6±3* a	7±2* a	10±2** a
BS/TV [mm^{-1}]	6.0±0.2	0.2±0.6 a	0.6±0.1* a	0.4±0.1* a	1.3±0.3* a
Tb.Th [mm]	0.21±0.01	0.06±0.05 a	0.01±0.01 a	0.03±0.020 a	0.01±0.01 a
Tb. Sp [mm]	0.41±0.02	−0.05±0.2* a	−0.05±0.01* a	−0.02±0.01 a	−0.06±0.02* a
Tb.N [mm^{-1}]	1.83±0.02	0.06±0.31** a	0.19±0.05*** a	0.05±0.11*** a	0.37±0.12*** a

Table 7.4: Principal component analysis to determine which of the many cartilage and bone parameters were closely related to each other. Dominant factors are boldface.

	Metric	Factor 1	Factor 2
Cartilage Properties	Gross Score	**0.34**	−0.26
	Superficial Cellularity	−0.24	0.26
	Hexabrix Gray Value	0.23	0.00
	Material Stiffness	−0.23	0.22
OCA Graft Bone Properties	Structural Score	**0.35**	−0.10
	ScB BV/TV	−0.24	**0.33**
	ScB BS/BV	0.20	**−0.33**
	BV/TV	0.05	**0.40**
	BS/TV	−0.27	−0.13
	Tb.Th	0.24	0.30
	Tb.Sp	0.24	−0.15
	Tb.N	−0.31	−0.03
Peri-(Base) Cyst Wall Bone Properties	BV/TV	0.23	**0.35**
	BS/TV	0.25	0.19
	Tb.Th	0.13	0.27
	Tb.Sp	−0.22	−0.30
	Tb.N	0.18	0.17
	% variance explained	35.7	24.9
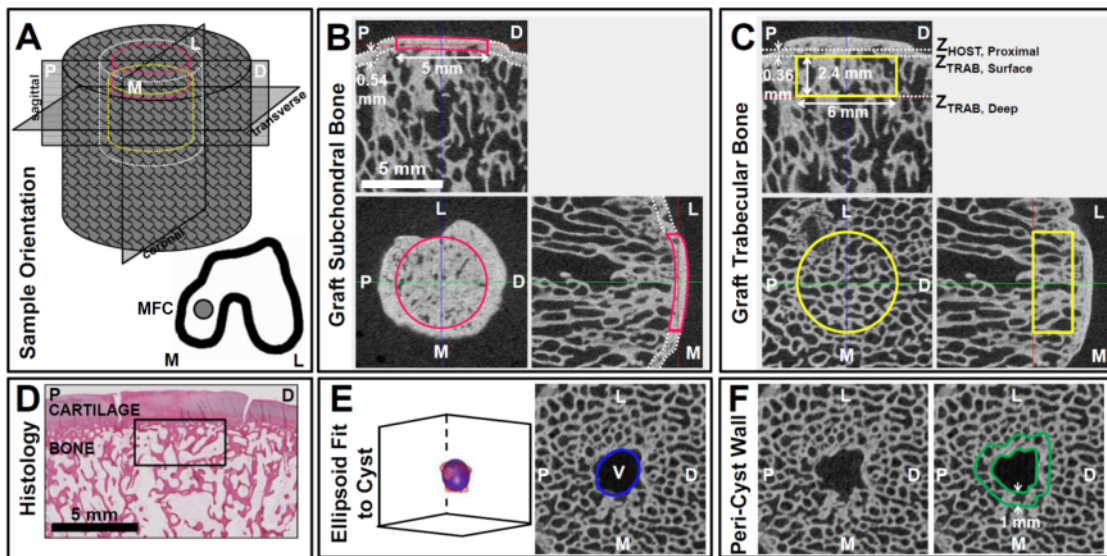
Figure 7.1: Sample orientation and definition of selected regions/volumes of interest (R/VOI). **(A)** Schematic diagram of sagittal, coronal, and transverse planes for osteochondral samples at medial femoral condyle (MFC). White dotted lines indicate allograft region in surrounding host bone volume. R/VOI locations for graft **(B)** subchondral and **(C,D)** trabecular bone, in addition to **(E)** subchondral bone cysts, fit to an ellipsoid to estimate volume (V), and **(F)** the peri-cyst wall defined on **(B,C,E,F)** typical μCT or **(D)** histology slices. P=proximal, D=distal, M=medial, L=lateral.
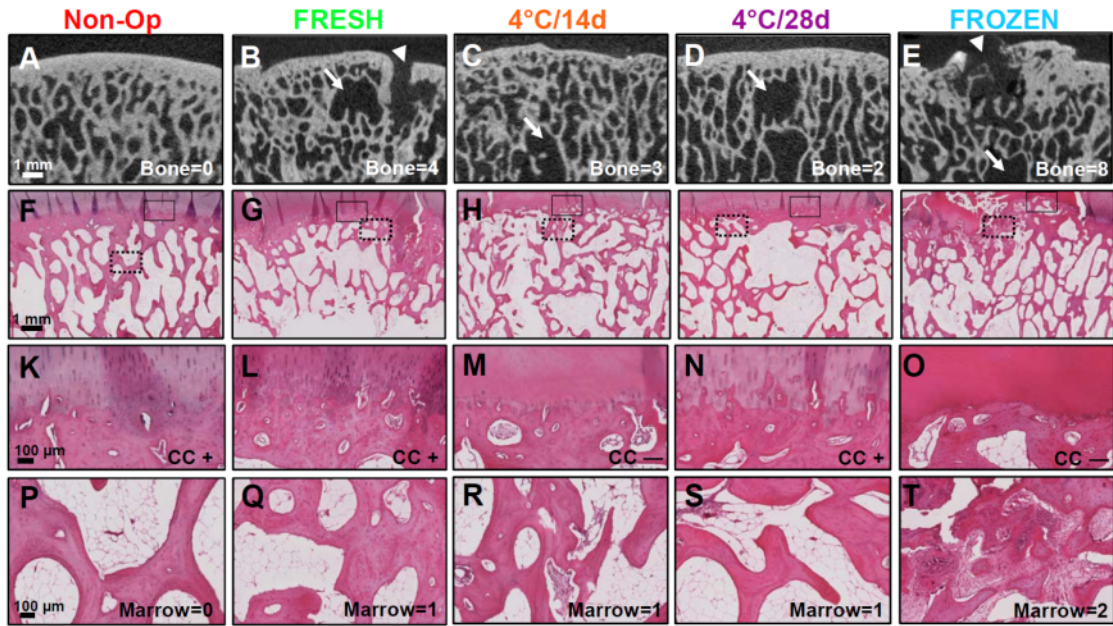
Figure 7.2: Effect of OCA storage on bone structure after 12 months *in vivo*. Representative **(A-E)** 2D μCT and **(F-T)** H&E bone histology images for **(A,F,K,P)** Non-Op, **(B,G,L,Q)** FRESH, **(C,H,M,R)** 4°C/14d, **(D,I,N,S)** 4°C/28d, and **(E,J,O,T)** FROZEN OCA. Bone cysts indicated by arrows, bone channels indicated by arrowheads. In F-J, solid box indicates zoomed region in K-O, dotted box indicates zoomed region in P-T. Values in the lower right corner correspond to the overall structural bone score, calcified cartilage (CC) or bone marrow score of the representative image.
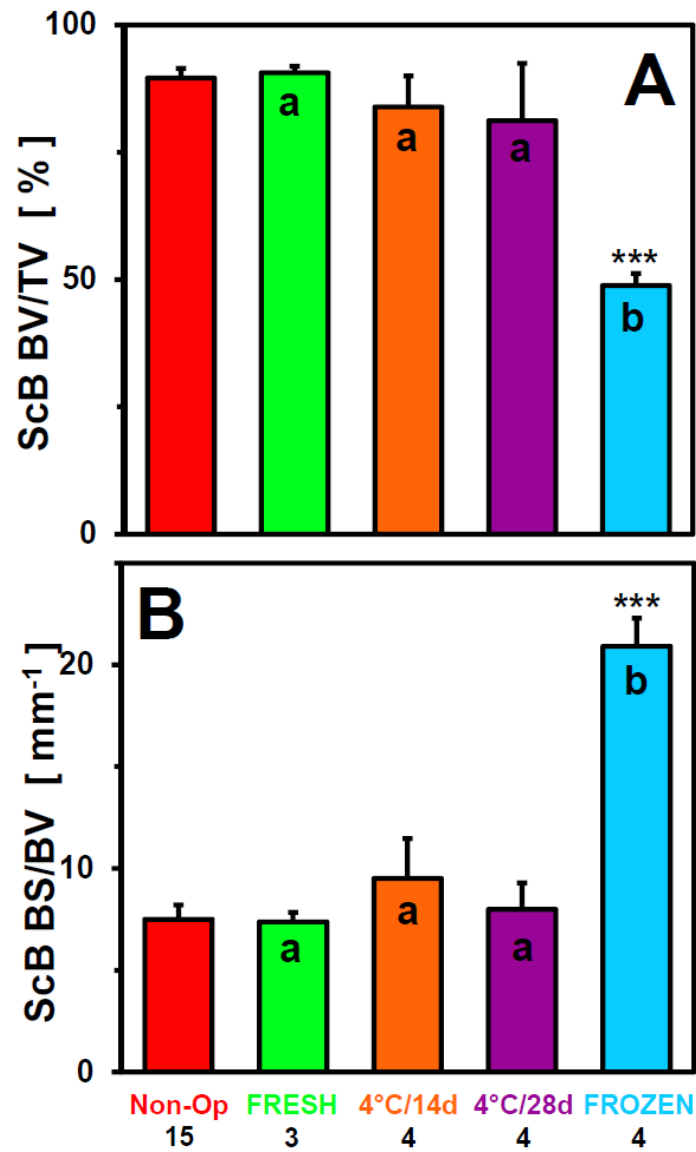
Figure 7.3: Effect of OCA storage on graft subchondral bone architecture after 12 months *in vivo*. Subchondral (ScB) **(A)** BV/TV [%] **(B)** BS/BV [mm^{-1}]. ***p<0.001 vs. Non-Op. Letters (a and b) designate significantly different groups (p<0.01), with a shared letter indicating no difference.
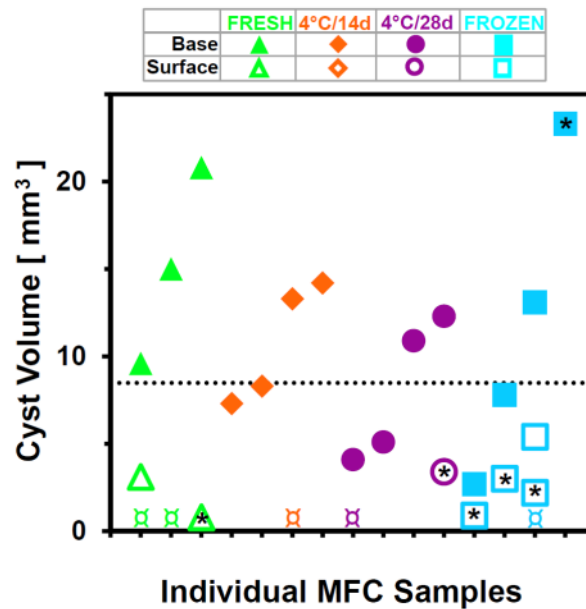
Figure 7.4: Volume of bone cysts within OCA after 12 months *in vivo*. Data points correspond to individual cysts for FRESH (green triangles), 4°C/14d (orange diamonds), 4°C/28d (purple circles), and FROZEN (blue squares) OCA. Open symbols indicate cysts near the bone-cartilage interface (i.e. "surface"), and filled symbols indicate cysts in deeper regions of the sample (i.e. "basal"). Dotted line indicates the average cyst volume (n=22). * indicates channel connects that cyst volume to the cartilage. ¤ indicates channel is present, but does not directly connect to a cyst.
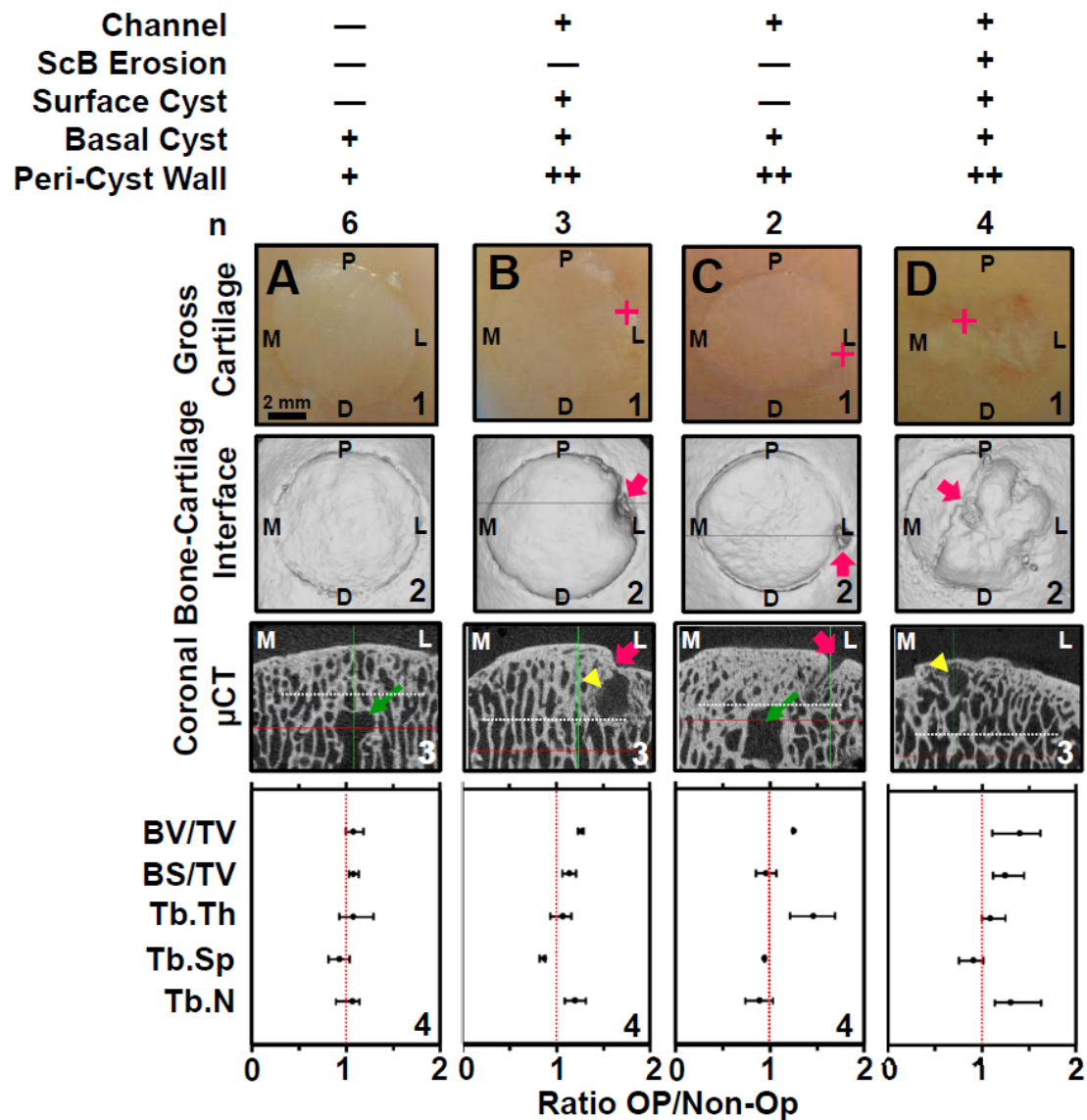
Figure 7.5: Characterization of bone cysts within OCA after 12 months *in vivo*. OCA had (**A**) basal cysts only, (**B**) both basal and surface cysts with surface channels, (**C**) basal cysts with surface channels, and (**D**) both basal and surface cysts with surface channels and subchondral bone (ScB) erosion. Representative images of (**1**) gross cartilage, (**2**) 3D renders of bone-cartilage interface, and (**3**) 2D coronal μCT slices. (**4**) Peri-(basal)cyst wall architecture relative to corresponding Non-Op, represented as mean and range. Plus signs and thick arrows in pink indicate surface channels. Thin arrows in green indicate base cysts. Arrowheads in yellow indicate surface cysts. Dotted lines in white indicate ~boundary between graft and host bone. P=proximal, D=distal, M=medial, L=lateral.
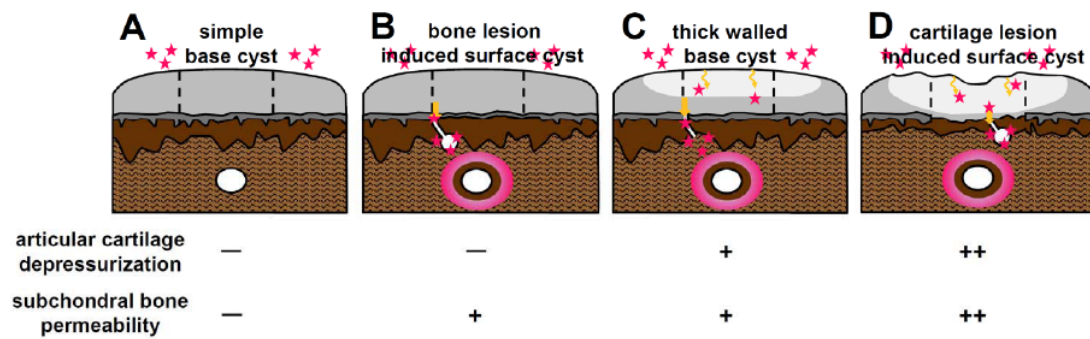
Figure 7.6: Schematic diagram hypothesizing development of bone cysts following OCA repair. Cyst structure may be (**A**) simple or (**C**) thick-walled in deeper "base" regions, or induced by (**B**) bone or (**D**) cartilage lesions at the surface. Plus and minus signs indicate magnitude of articular cartilage depressurization and subchondral bone (ScB) permeability, which may lead to the development of bone cysts.

7.7   Supplement

Supplementary tables figures, cited in the text, report examples of bone surface channels and histological analysis of bone cysts. For detailed 3D surface renders of the bone-cartilage interface, indicating bone surface channels, see Figure 7.S1. For histological analysis of base and surface cysts see Figure 7.S2.
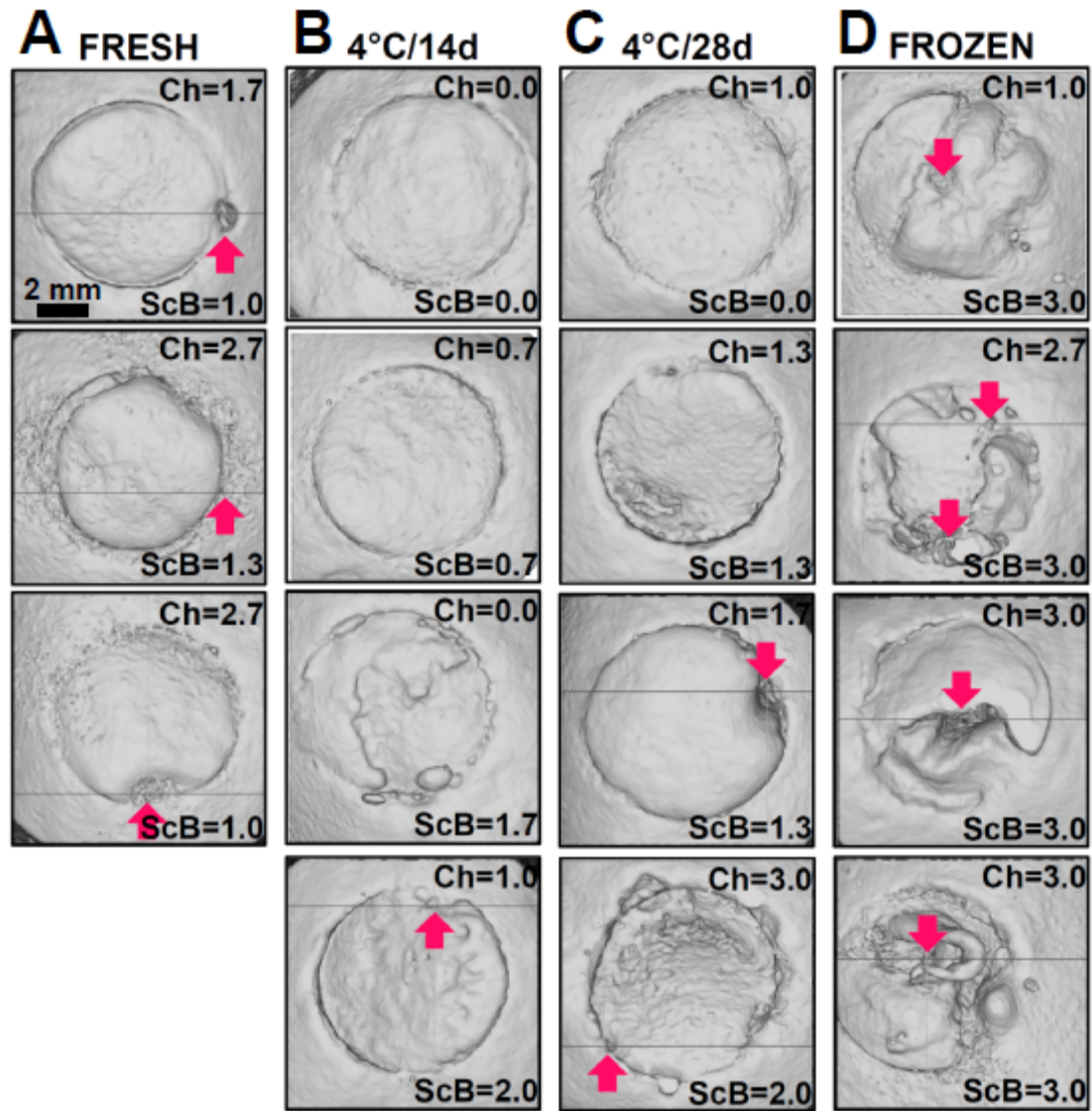
Figure 7.S1: Effect of OCA storage on bone channel size and bone-cartilage interface roughness after 12months *in vivo*. 3D renders of bone-cartilage interface for (**A**) FRESH, (**B**) 4°C/14d, (**C**) 4°C/28d, and (**D**) FROZEN OCA. Values in the upper and lower right corner correspond to the bone channel (Ch) score or subchondral bone (ScB) surface roughness score (each from 0-3) of that image. Thick arrows in pink indicate bone surface channel.
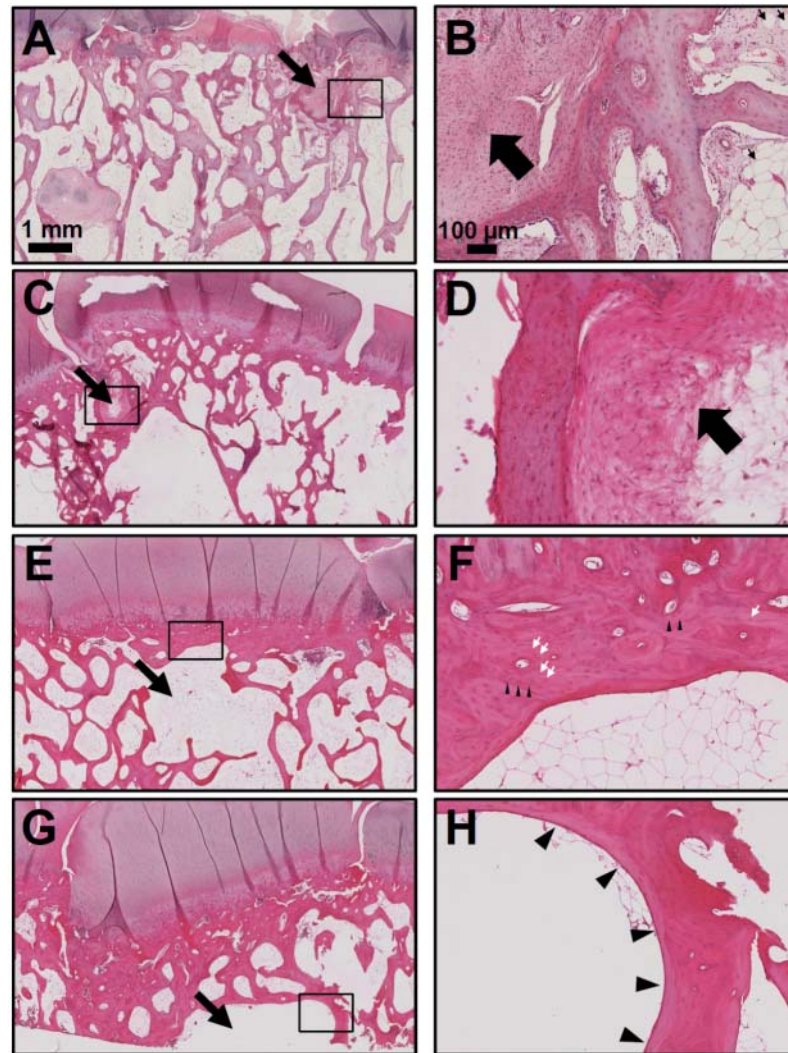
Figure 7.S2: Histologic appearance of selected bone cysts. (**A-D**) Surface cysts, which were associated with a bone surface channel, contained fibrous and collagenous tissue. (**E-H**) Other "basal" cysts contained normal marrow with primarily fat cells. In **A,C,E,G,** arrows indicate cysts while the solid box indicates zoomed region in **B,D,F,H**. In **B** and **D**, thick arrows in black indicate fibrous contents. In **B**, thin arrows in black indicate swollen fat cells and bone marrow edema. In **F**, arrow heads indicate reversal, while arrows in while indicate necrosis. In **H**, arrow heads indicate sclerotic bone.

7.8 References

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CHAPTER 8:

CONCLUSIONS

8.1   Summary of Findings

This dissertation developed an integrative and multi-scale approach to cartilage defect repair analysis by osteochondral allografts, investigating the effects of osteochondral storage on *in vivo* cartilage and bone remodeling. The studies presented in the preceding chapters were conducted 1) to validate the goat model of osteochondral allografting, 2) to examine the role of decreased cartilage cellularity during allograft storage on long-term *in vivo* cartilage repair outcomes, and 3) to identify potential biophysical and biological mechanisms of bone cyst formation following cartilage repair.

Systematic, multi-scale, and inter-disciplinary analysis validated the large animal model of osteochondral allografting and demonstrated the superiority of fresh versus frozen allografts (**Chapter 2**). The cartilage of fresh allografts maintained its zonal organization of cartilage cellularity and matrix content and load-bearing function similar to non-operated cartilage. In contrast, the cartilage of frozen allografts displayed signs of failure at 6months based on softening and associated deterioration of structural indices, particularly cellularity, deep zone fixed charge, and surface irregularity. While all allografts exhibited bony union at the graft-host junction, both frozen and fresh allografts were variably accompanied by structural abnormalities of bone healing, including marrow fibrosis and subchondral bone cysts. Site- and

possibly loading-dependent factors may influence *in vivo* repair outcomes as allografts failed differently at MFC and LT sites, with graft subsidence at MFC, but not at LT.

The associated decline in surface cellularity with overall tissue properties in retrieved osteochondral allografts suggests that 4°C stored allografts are somewhat susceptible to tissue degeneration after 12months *in vivo* (**Chapter 4**). Repair outcomes in 4°C stored allografts were similar after 14d and 28d storage, and both were inferior to fresh allografts, accompanied by diminished cellularity at the surface, matrix fixed charge, and histopathological structure. In allografts stored at 4°C for 14d and 28d, cellularity was diminished by ~60% within the first 50µm of the articular surface versus non-operated and fresh cartilage, identifying a region vulnerable to storage-associated cell death. Cartilage macroscopic gross-score in fresh allografts was consistently low at MFC, but more variable at LT and similar to allografts stored at 4°C for 14d and 28d. Indices of cartilage health, including gross-score, histopathology-score, matrix fixed charge, and cartilage stiffness, correlated significantly with superficial cartilage cellularity.

The PRG4-secreting function of allografts was maintained *in vivo* based on its state after osteochondral storage (**Chapters 3 and 5**). PRG4 secretion from cartilage after osteochondral storage at 4°C, especially after 28days, was lower than fresh allografts, and similar to frozen allografts. After 6months *in vivo*, PRG4 secretion of cartilage and lubricating function of cartilage conditioned medium from frozen allografts was markedly reduced versus Non-Op and fresh allografts. After 12months *in vivo*, PRG4 secretion of cartilage from stored allografts tended to decrease with increasing 4°C storage duration. PRG4 secretion was a useful indicator of *in vivo* allograft performance, as indices of *in vivo* repair efficacy, including gross-score,

histopathology-score, superficial cellularity, and cartilage stiffness, correlated significantly with subsequent PRG4 secretion in retrieved allografts at 12months.

Alternative storage conditions, such as 37°C storage, support long-term chondrocyte viability, especially at the vulnerable articular surface (**Chapter 6**). 37°C storage maintained high chondrocyte viability compared to traditional 4°C storage for prolonged intervals. The results presented here quantitatively assess chondrocyte viability at the articular surface *en face* and in morphometrically analogous cartilage zones. Cartilage thickness and matrix content were maintained for 37°C and 4°C stored osteochondral samples compared to fresh controls. Storage of allografts at physiological temperature of 37°C may prolong osteochondral donor storage duration, increase graft availability, and improve treatment outcomes.

Finally, subchondral bone deterioration in frozen allografts, in association with degeneration of the articular cartilage, suggested that cartilage and bone coupling may affect biological and structural remodeling during osteochondral repair, and various biophysical mechanisms can be postulated based on structural aspects of the bone state at retrieval (**Chapter 7**). Bone structure in stored allografts after 12months *in vivo* was altered compared to non-operated joints, with allografts displaying bone cysts and bone surface channels. Bone structure in frozen allografts was worse than fresh and 4°C stored allografts, with severe subchondral bone erosion and infiltration of fibrous tissue into the bone marrow, which may have implications for cyst etiology. Subchondral bone cysts were widespread in allografts, with some cysts in the deeper regions of the graft (i.e. "base" cysts), and other (fewer and smaller) cysts near the bone-cartilage interface (i.e. "surface" cysts). While trabecular bone architecture in the graft and peri-(base)cyst region was altered in allograft versus non-operated bone, with allografts generally having higher bone volume fraction, identification of patterns

of bone cyst structure were used to categorize and differentiate allografts with different pathological states of bone remodeling. Bone cyst pathogenesis may be due to inadequate osseous integration, or induced by either cartilage or bone lesions.

8.2   Discussion

Major contributions of the current work include: extension of the current paradigm that 4°C stored allografts have decreased chondrocyte viability *in vitro* to include the effects of 4°C storage on diminished *in vivo* performance; identification of potential biomarkers of allograft performance and alternative storage protocols that enhance and extend chondrocyte viability *in vitro*; insight into the pathogenesis of subchondral bone cysts during cartilage defect repair; and development of an integrative analysis of cartilage defect repair that highlights the interrelationship between biological and structural aspects of cartilage and bone remodeling *in vivo*. Further mechanistic studies detailing the progression of bone cysts are needed; however, preliminary observations in this dissertation have laid the groundwork to focus future hypothesis-driven studies. Clinical implications of this work include the need to 1) shorten 4°C storage duration of donor osteochondral allografts, 2) utilize alternative storage conditions which preserve chondrocyte viability, and 3) screen allografts prior to implantation in order to improve patient outcomes.

While the goat model used in these studies provided a useful large animal model for studying osteochondral defect repair as it exhibits limited intrinsic healing [1], translation to the clinical situation may be dependent on several factors. *In vitro* viability data of goat tissue were similar to viability data of human tissue following clinically established 4°C storage conditions [22, 23]; however, the metabolic properties of adult goat cartilage during storage may differ compared to human cartilage, especially at physiological temperature, and *ex vivo* maintenance of glycosaminoglycan content at the joint-scale may require additional anabolic stimuli or catabolic inhibitors [14]. Since goat cartilage is thinner and more cellular than human cartilage [1, 6], *in vivo* repair efficacy in humans may be related to an absolute

minimum number of cells rather than diminished regional cell density important in the goat model, as similar decreases in cell density (i.e. $\Delta=5\times10^6$ cells/cm^3) between human and goat cartilage would represent a larger percentage of dead cells in human cartilage. While widespread occurrence of subchondral bone cysts is not well-documented clinically, repair outcomes in patients are affected by effective osseous integration, can be interrelated between cartilage and bone properties, and prevention of bone cysts would improve allograft efficacy.

During this work, a novel, integrative, multi-disciplinary, and multi-scale approach was taken to analyze cartilage defect repair. The endpoints measured encompassed both structural and biological parameters of cartilage and bone, measured by a variety of traditional (biomechanical, histological, and biochemical) and cutting-edge (micro-computed tomography, confocal microscopy) technology. Since data from human allograft retrievals is skewed toward poor outcomes (i.e. analysis is performed on "failed" cases) [9, 19, 29], using systematic analysis in animal models is critical to elucidate tissue changes of early stage degeneration, and to provide insight into the time-course of cartilage remodeling and/or deterioration. Previous large animal studies of osteochondral allograft repair, typically limited to gross and histological evaluation, with few studies including biomechanical analysis, of cartilage [2, 7, 12, 20, 21, 24-27], failed to identify determinants of cartilage deterioration or maintenance, and ignored possible implications from the underlying bony support. Analyzing cartilage and bone biology, structure, and function in an integrative manner identified properties of the osteochondral tissue that are critical to repair efficacy and potential mechanisms of graft success/failure, and could also be applied to other cartilage repair and tissue-engineered therapies.

The results obtained here, using such systematic and integrative analysis described above, have contributed to the understanding of the role of decreased cartilage cellularity during allograft storage on long-term *in vivo* cartilage repair outcomes. The associated decline in surface cellularity with overall tissue properties in retrieved osteochondral allografts suggests that 4°C stored grafts are inferior compared to fresh allografts, which have consistently good outcomes at MFC and serve as a baseline for comparison; however, 4°C stored grafts may still provide reasonable clinical outcomes versus pre-operative function. Surgeons question the effectiveness of allografts stored at 4°C for prolonged durations (i.e. near the ~28d shelf-life) versus shorter durations (i.e. near the minimum ~14d screening and processing time), and while short-term clinical outcomes have not been linked to extended storage duration [11, 15, 28], the studies in this work suggest that minimizing the storage interval would be advantageous. The variability in cellularity after extended 4°C storage durations may require screening of grafts for a suitable level of chondrocyte viability prior to implantation; however, suitable cell density in the goat model may not be similar in humans.

The high rates of PRG4 secretion, in association with surface cellularity and *in vivo* repair outcomes, for fresh allografts suggests that PRG4-secreting function may be a useful marker of biological function following cartilage repair. While biomarkers, characteristics that objectively measure and evaluate normal biological processes, have been studied extensively for osteoarthritis [10], biomarkers for pre-clinical models of cartilage repair have not. PRG4, a mechanosensitive indicator of cartilage surface health [4, 16-18, 30] that is typically secreted by superficial zone chondroctyes, may be a suitable candidate since surface cellularity is critical to osteochondral allograft repair efficacy. Since PRG4-secreting function of allografts

appears to be maintained in vivo based on its state after storage, allograft screening for PRG4 secretion may be able to define high quality allograft tissue suitable for implantation.

Structural and histological abnormalities in bone healing within allografts may be related to the state of cartilage within osteochondral allografts. Structural changes and an increase in the ease of fluid transport through the osteochondral interface in osteoarthritic cartilage suggest that the subchondral bone plate is an important mediator of interactions between cartilage and bone [8]. In the etiology of subchondral bone cysts, fluid transport through bone channels and/or cartilage depressurization across the subchondral bone plate may cause altered biological and mechanical signals to be transmitted to the underlying bone, and result in abnormal bone structure. Strong relationships between cartilage and bone structure were evident from the integrative analysis of cartilage defect repair. Clinically translatable endpoints, including imaging results from computed tomography and magnetic resonance imaging, would be useful to extrapolate the results from the goat model to patients, and allow for longitudinal studies on cyst development to be analyzed.

Taken together, the results of this dissertation work have contributed to a further understanding of *in vivo* cartilage and bone remodeling after cartilage defect repair by osteochondral allografts. The findings reported here are consistent with the overall hypothesis that the biological and structural properties of the osteochondral allograft implant affect the efficacy of functional repair due to the mechanobiology of the articular surface, preservation of the cartilage-bone interface, and the integration of graft bone with host bone. Maintenance of a smooth articular surface, fixed-charge-rich bulk cartilage, intact subchondral bone plate, and appropriately remodeled bone structure are likely key regulators of *in vivo* allograft performance.

8.3   Future Work

The current work can be expanded upon in a number of ways, including applying this integrative and multi-scale analysis to other cartilage repair therapies or tissue-engineered osteo-cartilaginous constructs, assessing the long-term *in vivo* performance of 37°C stored allografts, and improving osseous integration during osteochondral allograft repair by mechanistically studying the development of bone cysts and proposing interventional strategies.

In the current work, surface cellularity and bone surface channels were identified through the integrative and multi-scale analysis of cartilage defect repair to be critical in repair efficacy of the cartilage and bone components. Investigating other cartilage repair strategies or tissue-engineered constructs using a similarly systematic and rigorous analysis method may help to elucidate factors that are essential for the success of the selected treatment. In addition, the integrative analysis described here could be expanded to encompass clinically translatable endpoints, including imaging studies with computed tomography (CT) and magnetic resonance imaging (MRI) [5]. Such non-invasive imaging analysis of osteochondral allografts, in association to the destructive structural and biological analyses outlined here, would provide further extrapolation to the clinical scenario, allow for longitudinal studies, and may identify allografts that could be prone to degeneration/failure at early stages.

A new storage paradigm or screening procedure for osteochondral allografts is needed that leads to improved overall allograft efficacy. While alternative storage conditions, such as 37°C storage of osteochondral grafts, preserve chondrocyte viability *in vitro*, the long-term efficacy of such grafts remains to be elucidated. 37°C stored allografts may also have better biological performance than 4°C stored allografts. Alternatively, PRG4 secretion could be measured from either 37°C or 4°C

grafts prior to implantation to select grafts with the greatest potential for good repair outcomes. It would be interesting to determine the *in vivo* performance of 37°C vs. 4°C stored allografts, especially if some 4°C stored grafts (i.e. ones that maintained higher viability than average) had similar PRG4 secretion to 37°C stored grafts. It is possible that PRG4 secretion in 37°C allografts may plateau during *in vitro* storage/screening, but the cells are not damaged and could resume PRG4 secretion *in vivo* compared to 4°C stored allografts, whose cells may have active esterases after storage (i.e. stain positively with Live/Dead), but whose metabolism is irreparably compromised and cannot be stimulated *in vivo*. It would also be interesting to determine if 37°C storage affects osseous integration, as 4°C stored allografts are believed to be less immunogenic that fresh allografts [3].

Given the association between bone surface channels, fibrous marrow, and subchondral bone cysts in osteochondral allograft repair, it would be interesting to elucidate the mechanism of cyst development and propose interventional strategies to improve osseous integration. Since "base" bone cysts were observed in all allografts at the MFC, it is possible that one mechanism of cyst formation is related to the structure of the implant and host geometry. One hypothesis suggests that inadequate load transfer across the base of the allograft leads to bone resorption and the formation of bone cysts. While it is challenging to measure bone deformation *in vivo*, an *in vitro* model of osteochondral allografts at implantation could be analyzed during loading by micro-computed tomography to determine the deformation of trabeculae in various graft fits. Alternatively, "surface" cysts were observed in conjunction with subchondral bone degeneration, bone surface channels, or severe cartilage degeneration; it is possible that another mechanism of cyst formation is related to cartilage depressurization forcing excessive fluid into the underlying bone and

resulting in abnormal bone biology. Examining the hydraulic conductance (ease of fluid flow) by perfusion-testing [8, 13], as a functional index of fluid-pressurizing ability of the osteochondral unit would be useful to examine mechanobiological mechanisms of bone healing. Elucidating the mechanism for inadequate osseous integration and cyst formation following cartilage repair would be useful to identify potential therapeutic interventions and improve the overall efficacy of osteochondral allografts.

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