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Mechanisms of articular cartilage defect repair in vivo after implantation of stratified cartilaginous tissue

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MECHANISMS OF ARTICULAR CARTILAGE DEFECT REPAIR \textit{IN VIVO} AFTER IMPLANTATION OF STRATIFIED CARTILAGINOUS TISSUE

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Kanika Chawla

Committee in charge:

Professor Robert L. Sah, Chair
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Professor Willian D. Bugbee
Professor Shu Chien
Professor Koichi Masuda
Professor Gabriel A. Silva

2006
The dissertation of Kanika Chawla is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2006
# TABLE OF CONTENTS

Signature Page ............................................................................................................. iii

Table of Contents......................................................................................................... iv

List of Figures and Tables .......................................................................................... ix

Acknowledgments....................................................................................................... xii

Vita.................................................................................................................................. xviii

Abstract......................................................................................................................... xx

Chapter 1: Introduction .................................................................................................. 1

1.1 General Introduction to the Dissertation .......................................................... 1

1.2 Structure, Composition, and Function of Articular Cartilage ....................... 4

1.3 Cartilage Damage and Current Repair Strategies ............................................. 9

1.4 Tissue-Engineered Cartilage ........................................................................ 12

1.5 Tracking Cells In Vitro and In Vivo ................................................................. 17

1.6 Overall Hypothesis: Early In Vivo Repair of Articular Defects ................. 21
# Chapter 2: Tracking Chondrocytes and Assessing Their Proliferation with PKH26: Effects on Secretion of Proteoglycan 4 (PRG4)

2.1 Abstract

2.2 Introduction

2.3 Materials and Methods

2.4 Results

2.5 Discussion

2.6 Acknowledgments

2.7 References

# Chapter 3: Tracking Chondrocytes and Assessing Their Proliferation with CFSE: Effects on Cell Function and Secretion of Proteoglycan 4 (PRG4)

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.4 Results

3.5 Discussion
Chapter 4: Short-term Retention of Chondrocytes in Stratified Tissue-Engineered Cartilaginous Constructs Implanted In Vivo in Mini-Pigs

4.1 Abstract

4.2 Introduction

4.3 Materials and Methods

4.4 Results

4.5 Discussion

4.6 Acknowledgments

4.7 References

Chapter 5: Stratification of Cartilaginous Tissue Implants with Labeled Chondrocyte Subpopulations: Effects on Early In Vivo Repair of Articular Defects

5.1 Abstract

5.2 Introduction

5.3 Materials and Methods

5.4 Results
Appendix B ................................................................................................................ 201

B.1 Introduction ................................................................................................. 201
B.2 Methods ..................................................................................................... 202
B.3 Results ....................................................................................................... 203
B.4 Discussion .................................................................................................. 211
B.5 References .................................................................................................. 213
LIST OF FIGURES AND TABLES

Figure. 1.1: Articular cartilage: length scales.................................................................7
Figure. 1.2: Zonal variations in articular cartilage.........................................................8
Figure. 1.3: Surgical treatment strategies for cartilage repair.......................................11
Figure. 1.4: Cell-based treatment strategies for cartilage repair.................................16
Table 1.1: Stability of cytotracking methods ...............................................................19
Figure. 1.5: Cytotracking methods .............................................................................20
Figure. 1.6: Proposed hypothesis for early in vivo repair of articular cartilage defects by implantation of cell-laden tissues .......................................................22
Figure 2.1: Schematic of methods used to evaluate retention of PKH26.....................39
Figure 2.2: Effects of cell growth and seeding density on PKH26 retention..............44
Figure 2.3: Effects of seeding density and time in monolayer culture on PKH26 retention...................................................................................................46
Figure 2.4: Generation model for cells labeled with PKH26......................................47
Figure 2.5: Effects of chondrocyte origin and seeding density on proliferation............49
Figure 2.6: Effects of chondrocyte origin and seeding density on PKH26 retention...52
Figure 2.7: Effects of cell type, tagging, and cell density on relative proliferation and PRG4 secretion........................................................................................53
Figure 3.1: Schematic of methods used to evaluate retention of CFSE .......................67
Figure 3.2: Effects of cell growth and seeding density on CFSE retention ..............71
Figure 3.3: Effects of seeding density and time in monolayer culture on CFSE retention...................................................................................................73
Figure 3.4: Effects of chondrocyte origin and seeding density on proliferation..................75
Figure 3.5: Effects of chondrocyte origin and seeding density on CFSE retention.....77
Figure 3.6: Effects of cell type, tagging, and cell density on relative proliferation and PRG4 secretion........................................................................................79
Figure 3.7: Effects of cell type, tagging, and cell density on GAG secretion .......... 80

Figure 4.1: Schematic of methods used to generate stratified engineered constructs containing PKH26-labeled cells ............................................................... 96

Figure 4.2: Image processing of fluorescence micrographs of retrieved constructs .. 100

Figure 4.3: Construct implantation and retrieval in Yucatan mini-pig ...................... 103

Figure 4.4: Histological assessment of type I and II collagen in normal, implanted, and retrieved tissues ............................................................... 106

Table 4.1: Biochemical properties of cartilaginous tissues ........................................ 108

Table 4.2: Biomechanical properties of cartilaginous tissues ................................. 109

Figure 4.5: Fluorescence micrographs of normal, implanted, and retrieved tissues .. 111

Figure 4.6: Histological assessment of normal and retrieved tissues by H&E and Safranin O/FastGreen ............................................................... 114

Figure 4.7: Thickness and cellularity of implanted and retrieved tissues ............... 115

Figure 4.8: Percentage of PKH26-labeled cells detected at implantation and retrieval ..................................................................................... 119

Figure 5.1: Schematic of methods used to generate stratified and mixed tissue engineered constructs containing PKH26-labeled and CFSE-labeled cells ............................................................... 140

Figure 5.2: Construct implantation and retrieval in skeletally mature Yucatan mini-pigs. ....................................................................................................... 147

Table 5.1: Semi-quantitative grading scale for assessment of articular cartilage repair ........................................................................................................... 150

Figure 5.3: Effect of defect treatment and time on thickness and biochemical content ........................................................................................................... 154

Figure 5.4: Histological assessment of cartilaginous tissues, normal cartilage, and tissues filling empty defects ........................................................................ 155

Figure 5.5: Effects of defect treatment and time on type I and II collagen distribution ........................................................................................................... 156

Table 5.2: Biomechanical properties of $S^*/M^*$ and $S^*/M^*$ constructs at the time of implantation ............................................................... 157
Figure 5.6: Histological assessment of S*/M cartilaginous tissues at the time of retrieval..................................................................................................161

Table 5.3: Histopathological characteristics of samples. .................................................................162

Figure 5.7: Effects of construct type and defect treatment on surface roughness......163

Figure 5.8: Processed confocal images of samples to localized PKH26-labeled, CFSE-labeled, and other cells ..........................................................................166

Figure 5.9: Percentage of PKH26-labeled and CFSE-labeled cells recovered after 4 weeks in vivo ........................................................................................................167

Figure 6.1: Cellular fates in cartilage repair......................................................................................187

Figure A.1: Effects of cell type, BMP-7, and PKH26 labeling on biochemical composition ...................................................................................................................195

Figure A.2: Effects of BMP-7 on construct properties .................................................................196

Figure A.3: Effects of BMP-7 on stratification of S*/M constructs .............................................197

Figure B.1: Examples of samples analyzed for cellularity-interface bonded relationship .................................................................................................................................205

Figure B.2: Schematic of methods used to determine cellularity as a function of distance from the interface ..........................................................................................206

Figure B.3: Schematic of methods used to determine cellularity as a function of distance from the interface ..........................................................................................207

Figure B.4: Schematic of methods used to determine cellularity as a function of distance from the interface ..........................................................................................208

Table B.1: Cellularity values in the nearest bin to the interface ..................................................209

Figure B.5: Relationship between cellularity and interface bonded ........................................210
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ABSTRACT OF THE DISSERTATION

MECHANISMS OF ARTICULAR CARTILAGE DEFECT REPAIR IN VIVO
AFTER IMPLANTATION OF STRATIFIED CARTILAGINOUS TISSUE

by

Kanika Chawla

Doctor of Philosophy in Bioengineering
University of California, San Diego, 2006
Professor Robert L. Sah, Chair

Articular cartilage is a connective tissue that normally provides a load-bearing, low friction, wear-resistant tissue located at the ends of long bones through its depth-varying cell and matrix properties. When damaged, articular cartilage possesses limited capacity for self-repair. Cartilaginous tissue has been engineered to recapitulate the normal zonal organization of cartilage. The effects of such tissues as treatment for focal cartilage defects in vivo have not been determined. In addition, experimental studies have shown that implanted cells may not be retained in the defect site, and that host cells may also enter the defect site, both of which may modulate the efficacy of defect repair. This dissertation aims to establish cytotracking methods for chondrocytes and to use these methods to assess the cellular mechanisms of articular
cartilage defect repair *in vivo* after implantation of cartilaginous tissue that had a stratified population of cells resembling normal cartilage, or tissue that had a traditional, mixed population of cells.

Stratified and mixed cartilage constructs were formed with chondrocytes from the superficial and middle/deep zones of articular cartilage, fluorescently labeled with tracking dyes, PKH26 and CFSE. The dyes were strongly retained by chondrocytes, even when induced to proliferate at varying rates and were tracked through at least 7 cycles of cell division *in vitro*. The dyes were found to have minimal effects on chondrocyte function, including proliferation and secretion of PRG4.

Implant-derived cells were localized and recovered after implantation for 1 week however, stratification of cartilaginous tissues was not maintained *in vivo* suggesting contributions from both implant and host cells to the population of the defect. After 4 weeks *in vivo*, persistence of implant-derived cells in the defect region was observed with stratified constructs maintaining certain features of stratification after 4 weeks *in vivo* which were distinct from mixed constructs suggesting a role of cell organization and phenotype in cartilage repair. Clarifying cell source (implanted vs. host) and fates contributes to a mechanistic understanding of the repair of articular cartilage defects and possible tissue engineering repair strategies for the growing population of patients with impaired joint function.
CHAPTER 1

INTRODUCTION

1.1 General Introduction to the Dissertation

Articular cartilage is a connective tissue that normally provides a load-bearing, low friction, wear-resistant tissue located at the ends of long bones through its depth-varying cell and matrix properties. When damaged, articular cartilage possesses limited capacity for self-repair. A number of recent strategies for treating cartilage defects have relied on delivery of implanted cells to repair articular cartilage. One type of implant is cartilaginous tissue, formed from culture-expanded chondrocytes. Such tissue has been engineered to recapitulate the normal zonal organization of cartilage that has been generated in vitro, with cells in the superficial zone of these constructs secreting the lubricant molecule proteoglycan 4 (PRG4). The potential efficacy of these tissues for treating cartilage defects in vivo has not been determined. Experimental studies have shown that implanted cells may not be retained in the defect site, and that host cells may also enter the defect site, both of which may modulate the efficacy of defect repair. It is the overall aim of this dissertation to establish cytotracking methods for chondrocytes and to use these methods to assess the cellular mechanisms of articular cartilage defect repair in vivo after implantation of
cartilaginous tissue that had a stratified architecture of cells resembling normal cartilage, or tissue that had a traditional, mixed population of cells.

This chapter provides an introduction to the structure, composition, and function of articular cartilage as well as the current treatment strategies for joint repair including, biological surgical techniques and total joint replacement. Special attention is given to the emerging concept of repair using cartilage tissue engineering, with a focus on cytotracking methods, some of which were applied in this dissertation.

Chapter 2 details the use of a fluorescent tracker dye, PKH26, for labeling chondrocytes from different zones, establishes a method of using PKH26 for generation analysis of chondrocytes, and determines the effects of PKH26 on certain aspects of cell function, primarily proliferation and secretion of PRG4 protein. PKH26-labeled chondrocytes tracked through at least 7 cycles of cell division with minimal effects on cell proliferation and secretion of PRG4.

Similar to the study described in Chapter 2, Chapter 3 establishes the use of another fluorescent tracker dye, CFSE, for eventual labeling of chondrocytes seeded as part of dual-labeled cartilage constructs composed of multiple chondrocyte subpopulations. Chapter 3 establishes a method of using CFSE for generation analysis of chondrocytes, and investigates the effects of CFSE on proliferation, matrix content of cells, and secretion of PRG4 protein. CFSE-labeled chondrocytes were tracked in vitro with minimal effects on proliferation, matrix content, and secretion of PRG4.

Chapter 4 described the generation and characterization of cell-seeded stratified cartilaginous constructs with one or both chondrocyte subpopulations labeled with PKH26, the dye described in Chapter 1. The constructs were implanted in
vivo in a mini-pig animal model in order to determine the degree to which the stratified cartilaginous constructs maintain their architecture in a full thickness defect in vivo after implantation in the patellofemoral groove (PFG) for 1 week. Constructs were characterized biochemically, histologically, and biomechanically, and stratification visualized and quantified, before and after implant in order to determine persistence and organization of implanted (labeled) cells. Implant-derived cells were localized and recovered after implantation for 1 week however, stratification of cartilaginous tissues was not maintained in vivo suggesting contributions from both implant and host cells to the population of the defect.

Chapter 5 extends the findings of Chapter 4 by increasing the in vivo duration to 4 weeks and explores the effects of treating cartilage defects with constructs either stratified like normal cartilage, with the superficial cell population labeled with PKH26 and the middle cell population labeled with CFSE, or composed of a mixed population of superficial and middle cells, or no implant, on the early in vivo repair of articular cartilage defects. The depth-associated distribution and density of implant-derived cells are compared, as well as the quality of repair, as assessed by a semi-quantitative histological grading scale. Relationships between implant-derived cells and repair quality are also discussed. Interactions between host and implant appeared to be present which may affect cell retention and infiltration. Stratified constructs maintained certain features of stratification after 4 weeks in vivo which were distinct from mixed constructs suggesting a role of cell organization and phenotype in cartilage repair.
Finally, Chapter 6 summarizes the findings of the dissertation and discusses potential future directions for tissue engineered cartilage in repairing focal cartilage defects. Additional experiments are described to extend the research and determine functional benefits of the approach. Other approaches for cartilage tissue engineering are also presented.

1.2 Structure, Composition, and Function of Articular Cartilage

Articular cartilage normally functions as a low friction, wear-resistant, load-bearing tissue located at the ends of long bones in synovial joints [78, 79]. It consists primarily of chondrocytes embedded in a highly hydrated matrix composed of collagens (mainly type II), proteoglycans (mainly aggrecan), and other proteins [79] (Fig. 1.1). Cartilage contains aggrecan, a large molecule consisting of a core protein and a large number of negatively charged sugar moieties (glycosaminoglycans, GAG), primarily keratan sulfate (KS) and chondroitin sulfate (CS). These chains are highly negatively charged due to the presence of sulfate and carboxylate groups and thus, repel each other and cause swelling at physiological pH. This, in combination with the existing collagen network, causes cartilage to possess high tensile and shear stiffness and withstand high contact stresses in vivo [74].

The cell phenotype and matrix properties of articular cartilage vary with depth from the articular surface, creating superficial, middle, and deep regions with specialized functional properties [70] (Fig. 1.2). In the superficial zone, which corresponds to the uppermost 10% of the total cartilage thickness, the cells have a flattened morphology, are organized in clusters parallel to the articular surface [99],
and exist at a high cell density compared to deeper zones (human femoral condyle: ~24 million cells/cm$^3$ in superficial zone, ~8 million cells/cm$^3$ in deeper zones [44]). The superficial zone is also thought to contain progenitor cells [28]. Cells from the deeper zones of cartilage proliferate at a greater rate compared to cells from the superficial zone [2, 41]. Collagen fibrils are also oriented parallel to the surface and GAG content is low relative to deeper regions [70]. The middle or transitional zone corresponds to the next 50% of tissue depth and consists of randomly distributed spherical chondrocytes and collagen fibrils. GAG content in this zone is greater than in the superficial zone [24, 41, 101, 111]. The deepest portion of the cartilage thickness consists of columnar cells of increased size [41], collagen fibers perpendicular to the articular surface, high GAG content, and presence of specific molecules including collagen X [32]. The deep zone of cartilage integrates with calcified cartilage and is separated from the underlying bone by the tidemark.

The zonal organization of cartilage has implications for the biomechanical properties of the tissue. The compressive modulus of cartilage increases with depth from the articular surface and directly correlating with GAG content [92]. Collagen fibrils are parallel to the articular surface in the superficial region, providing the region with high tensile strength, distributed randomly in the middle, and perpendicular to articular surface in deeper regions [6]. Boundary lubricants encoded by the gene PRG4 [50] include the protein products Superficial Zone Protein (SZP) [96, 97], secreted by chondrocytes of the superficial zone [96]; synoviocytes [96, 97], and meniscal cells [98]; lubricin [53], abundant in synovial fluid [105]; megakaryocyte stimulating factor [55]; and proteoglycan 4 (PRG4). These highly homologous
molecules are thought to contribute to the low friction properties of articular cartilage (coefficient of friction 0.002-0.02) and can collectively be referred to as PRG4. The role of PRG4 as a boundary lubricant is supported by its presence at the surface of articular cartilage [96], its abundance in synovial fluid, its mutated form resulting in camptodactyly-arthropathy-coxa vara pericartitis syndrome [68], and its reduction of the friction coefficient when applied between natural and artificial materials [52, 54, 93].
Figure. 1.1: Articular cartilage: length scales. (A) Human knee joints are (B, C) covered with articular cartilage, which is comprised of (D) chondrocytes sparsely embedded in extracellular matrix, largely consisting of (E) proteoglycans and collagen. Cartilage in the body is hydrated in the synovial fluid. (Photo is provided by TJ Klein. Micrographs and collagen schematic were adapted from [16, 26, 45]).
**Figure. 1.2:** 3-dimensional histology of adult human articular cartilage, with demarcations of the classical zones and features thereof. (Adapted from [58]).
1.3 Cartilage Damage and Current Surgical Repair Strategies

Articular cartilage has a limited capacity for self-repair, likely due to its lack of vascularity and the sparsity of cells present [15, 67]. Unfortunately, cartilage is often diseased or injured due, in part, to aging and/or incurred trauma. More than 140 forms of arthritis afflict 66 million Americans, costing the U.S. economy $86.2 billion per year [20]. Osteoarthritis (OA), the most common degenerative joint disease, begins with superficial fibrillation [77] and can propagate to weakened cartilage reduced in GAG content [9] and collagen integrity [103]. Eventually, full thickness lesions and/or complete lack of cartilage can occur, resulting in regions of painful bone-bone contact.

Several existing surgical repair strategies are aimed at treating damaged joints (Fig. 1.3). Less invasive methods aim to debride damaged tissue or induce self-repair by penetrating the bone and causing the defect to fill with bone marrow derived cells in a fibrin clot [100, 104]. The repair tissue which forms is fibrous in nature and unlike normal cartilage, contains large amounts of type I collagen and low amounts of aggrecan and type II collagen. This tissue exhibits superficial fibrillation, abnormal integration with surrounding host tissues, and may eventually fail due to abnormal extracellular matrix content and organization [27, 40, 100]. In addition, analysis of synovial fluid indicate the presence of molecular markers of degradation [94] as early as one week after the procedure [42]. Osteochondral autografts or allografts can be transplanted from cadaveric donors into recipient patients and generally have good clinical and function outcomes [33]. However, the donor supply of young, healthy cartilage is unpredictable and limited and may be compromised in terms of cell
viability [17]. In mosaicplasty, osteochondral allografts are obtained from a non-loadbearing site in the patient’s knee and transplanted in the damaged area. This method has had some success but has some limitations including creation of donor site morbidity, lack of integration at host-graft interfaces, and the size of the treatable defect [61]. Finally, total joint replacement has been applied in an attempt to restore joint function in patients. The most invasive of all the aforementioned surgical techniques, total joint replacement involves removal of whole joint surfaces and replacing the surfaces with implants made of biocompatible metals, ceramics, and/or plastics [34].
Figure. 1.3: Surgical treatment strategies for cartilage repair. (Image of artificial knee from New York Times [31])
1.4 Tissue-Engineered Cartilage

Recently, several investigators have sought to engineer cartilaginous tissue in vitro to implant and act as a cartilage patch. The methods typically involve combining various tissues, cells, and materials, culturing them in vitro with certain conditions, and eventually implanting the construct into a cartilage defect.

There are a variety of design criteria for generating tissue-engineered articular cartilage with the main goal being to restore and maintain normal cartilage function [88]. In particular, the implant should provide a low friction, wear-resistant surface, should fill the defect and be able to resist compression, and integrate with the surrounding tissue. In order to attain these characteristics, the mechanical maturity and structural organization of cartilage constructs generated for repair of articular cartilage defects have been explored. Specifically, fetal-like tissue constructs have been generated since these may more readily integrate with surrounding recipient cartilage but may need to remodel in order to withstand in vivo compressive loads [82, 87]. Tissue constructs with high compressive modulus have also been generated so that the construct can bear load immediately [71, 75]. The structural organization may play an important role in the success of a cartilage construct [86]. While constructs with homogeneous organization have been generated, more recently, some aspects of the structural organization of native cartilage have been recapitulated in stratified cartilage constructs created with a biomimetic approach [57, 59]. Finally, there are a variety of additional parameters that are likely necessary for appropriate tissue repair and reconstruction including implant geometry and biochemical properties [19].
Experimental and clinical studies of cartilage repair have examined a number of cell-based tissue-engineering methods for repairing focal cartilage defects (Fig. 1.4) [89]. Cells can be delivered directly into a cartilage defect by injection of cells alone [5] or, for example, under a periosteum flap as in the autologous chondrocyte implantation (ACI) method (Fig. 1.3) [14]. This approach has been used by Genzyme® Biosurgery, and is the only clinically-available cartilage tissue engineering procedure available in the U.S. In ACI, cartilage biopsies are obtained from a non-loadbearing location in the patient’s knee. Chondrocytes are enzymatically isolated and expanded several fold in vitro and then injected into a full-thickness defect covered by a periosteal flap obtained from the tibia. The success of the technically difficult, time-consuming, and costly (~$25,000 surgery [63]) surgery is controversial, with a recent study indicating similar patient outcomes between ACI and microfracture treatments [60]. A second generation ACI procedure (matrix-induced autologous chondrocyte implantation (MACI)) incorporates the use of a collagen matrix seeded with autologous cells in the implantation procedure [69].

In addition to cell injection techniques, other cell delivery methods have also been employed. Cells have been encapsulated within fibrin as a delivery vehicle [40], or within photopolymerizable hydrogel as a vehicle [29]. Cells can be delivered into a cartilage defect within carrier scaffolds cultured for short (hours to days) [83] or long (weeks to months) [69, 95] time periods, as a cartilaginous construct synthesized without a scaffold [36], or as a cartilaginous layer as part of a composite scaffold [8].

In addition to the method of implantation, the type of cells isolated can influence the articular cartilage repair. Progenitor or stem cells from bone marrow
[76], periosteum [30], fat [108, 109], as well as chondrocytes from various types of cartilage (articular, auricular, nasal) have been also used to generate engineered cartilage. Traditionally, cartilaginous tissues have been made from chondrocytes obtained from full thickness cartilage. However, phenotypic differences between superficial chondrocytes and cells from deeper zones indicate the likely importance of recapitulating the normal zonal architecture of articular cartilage.

In an effort to generate cohesive tissues for implantation, several steps are typically undertaken during *in vitro* culture. Initially, cells are expanded in number. In order to counteract the dedifferentiation of chondrocytes into a fibroblastic phenotype, which occurs during two-dimensional monolayer culture, chondrocytes have been induced to redifferentiate in three-dimensional culture systems [7]. In three-dimensional culture systems, chondrocytes can retain normal morphology and differentiated chondrocyte function. One method that particularly illustrates this is the alginate recovered chondrocyte (ARC) method [72]. In this method, chondrocytes are cultured in alginate to encourage formation of pericellular matrix. Cells with associated matrix are then released from the alginate by dissolving the gel. Cells cultured by this method appear to retain normal chondrocyte phenotype and form a cohesive tissue over time in culture [73]. Cells from the different zones of articular cartilage have been cultured in this manner and then used to form layered cartilaginous tissues [59] resembling some aspects of normal cartilage. Modulation of construct development and maturation *in vitro* may be further influenced by chemical and mechanical stimuli. Addition of growth factors to culture media and/or application of dynamic mechanical stimulation (compression, tension, shear, fluid flow) may
influence the generated engineered tissue along with the cell seeding density and duration [59], and hydrogel material [57] and concentrations.

A variety of animal models of cartilage repair are currently being used to investigate efficacy of cell-based therapies in vivo [46]. Research efforts vary in terms of the age and type of species chosen, the number, location (patellofemoral groove (PFG) or femoral condyle), type (partial chondral, full thickness chondral, or osteochondral), and size of articular cartilage defects, as well as the type of cell-based treatment applied, and the length of study. Large animal models, such as dogs [10-12], goats [51, 62], horses [40, 81, 90, 91], and pigs [21, 39, 47-49, 64, 66], as well as smaller animal models, i.e. rabbits [18, 107], and mice [56, 110] have been applied. In addition, multiple large (>5 mm) and small (<5 mm) chondral or osteochondral defects have been applied in both non-load bearing (PFG) and load bearing (femoral condyle) regions in the knee [12, 48, 80, 81, 102]. Translation of in vitro studies of cartilage repair to in vivo studies are challenging but necessary for investigating efficacy of putative tissue-engineering strategies [46].
Figure. 1.4: Cell-based treatment strategies for cartilage repair.
1.5 Tracking Cells *In Vitro* and *In Vivo*

Experimental studies have shown that implanted cells may not be retained in the defect site. Using various tagging methods and cell delivery methods, the number of recovered, tagged cells, relative to the number implanted, can be low as early as one day after the implant [83]. Recovery of implanted cells has been enhanced with pre-incubation of cells in carrier scaffolds [3], or incubation to pre-form tissue constructs however, it is likely that the cells at the defect are likely to be derived both from efflux of implanted cells and influx of host cells, in combination with various cellular fates, and this may modulate the efficacy of defect repair.

Existing methods for tracking chondrogenic cells *in vitro* and *in vivo* are variable in terms of tracer detectability at increasing follow up durations, as well as versatility of use and ability to distinguish cellular organization and cell populations (Table 1.1, Fig. 1,5). A variety of tracking probes can be applied for both short and long-term studies. Application of most fluorescent cytoplasmic dyes (CellTracker™ Green CMFDA, CellTracker™ Orange CMTMR, BCECF-AM), for tracking cells and determining cellular organization is limited to short-term studies lasting only a few days [38] due to dye dilution with cellular division. The dyes have been used to label chondrocytes [23] by introducing thiol-reactive fluorophores to the cell, which are cleaved by cytoplasmic esterases and become trapped in the cytoplasm of viable cells [38]. A longer-lasting cytoplasmic dye is carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) which passively diffuses into cells and is highly fluorescent and well-retained once its acetate groups are cleaved by intracellular esterases [84]. In
contrast, transduction of cells with a reporter gene such as β-galactosidase (lacZ) [4] or Green Fluorescent Protein (GFP) [43] allows for cell tracking as long as the gene is retained and expressed. However, transfection efficiencies of cells, especially in primary isolates or early passages can be technically difficult [65], while infection with viral vectors is also challenging, and can invoke an immune response [106]. Radioactive probes can also be useful for longer-term cell tracking depending on probe lifetimes. Tritiated thymidine [23] and the thymidine analog bromodeoxyuridine (BrdU) [13] can label newly synthesized DNA formed by chondrocytes. The latter has been detected after as long as 12 weeks in vivo [13]. However, determining the cellular organization of radiolabeled cells is difficult and quantitative analysis is limited to two-dimensional histological sections by autoradiography [35]. Alternatively, cell populations can be distinguished by intrinsic cell properties. The testis-sex determining region Y gene, SRY, which is present in cells originating from male, but not female, donors or recipients has been used in primary and expanded cells, and has been validated in studies up to 12 weeks [83]. Unfortunately, while the number of donor cells can be determined, the cellular localization of these cells is unknown [83]. For all of the above methods, it is difficult to assess the division history of the implanted cells. More recently, quantum dots have been applied for tracking of various cell types [1] but questions regarding quantum dot toxicity remain. While short-term dyes are useful for certain applications, ultimately, an efficient, stable fluorescent dye would be useful for assessing mechanisms of cartilage repair over a possible time scale of weeks to months.
Table 1.1: Stability of cytotracking methods.

<table>
<thead>
<tr>
<th>persistence</th>
<th>location</th>
<th>efficiency</th>
<th>tracker</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>days – wks</td>
<td>nucleus</td>
<td>&gt;95%</td>
<td>H33342</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;95%</td>
<td>BrdU, $^3$H-DNA</td>
<td>[13],[35]</td>
</tr>
<tr>
<td>wks</td>
<td>nucleus (genomic)</td>
<td>60-90%</td>
<td>GFP, lacZ</td>
<td>[5],[43]</td>
</tr>
<tr>
<td></td>
<td>cytoplasm</td>
<td>&gt;95%</td>
<td>CellTracker$^\text{TM}$</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;95%</td>
<td>CFSE</td>
<td>[37]</td>
</tr>
<tr>
<td>wks – months</td>
<td>endosomes/lysosomes</td>
<td>80%</td>
<td>quantum dots</td>
<td>[1]</td>
</tr>
<tr>
<td>months</td>
<td>cell membrane</td>
<td>&gt;95%</td>
<td>PKH26</td>
<td>[22],[25]</td>
</tr>
<tr>
<td>$\infty$</td>
<td>nucleus (genomic)</td>
<td>100%</td>
<td>SRY</td>
<td>[83]</td>
</tr>
</tbody>
</table>
Figure. 1.5: Cytotracking methods.
1.6 Early *In Vivo* Repair of Articular Defects

It is generally presumed that implant effectiveness depends on retention of implanted cells. Implanted cells, of the appropriate phenotype, can be retained in sufficient number and in an organized fashion in the defect in order to fill the defect with newly synthesized extracellular matrix and promote integration with the surrounding host tissues. The overall hypothesis of this dissertation is that early *in vivo* repair of articular cartilage defects is influenced by both the persistence of implanted cells as well as the depth-associated cell organization of superficial and middle cells in implanted constructs (Fig. 1.6).
Figure. 1.6: Proposed hypothesis for early *in vivo* repair of articular cartilage defects by implantation of cell-laden tissues
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CHAPTER 2

TRACKING CHONDROCYTES AND ASSESSING THEIR PROLIFERATION WITH PKH26: EFFECTS ON SECRETION OF PROTEOGLYCAN 4 (PRG4)

2.1 Abstract

Distinguishing between implanted and host-derived cells, as well as between distinct cell phenotypes, would be useful in assessing the mechanisms of cell-based repair of cartilage. The fluorescent tracker dye, PKH26, was previously applied to several cell types to assess proliferation \textit{in vitro} and to track cells \textit{in vivo}. The objectives of this study were to assess the utility of PKH26 for tracking chondrocytes from superficial and middle zones and their proliferation, and determine the effects of PKH26 on chondrocyte functions, in particular, proliferation and secretion of proteoglycan 4 (PRG4). PKH26-labeled and unlabeled superficial and middle zone chondrocytes were plated in either low or high density monolayer culture and analyzed for retention of PKH26 by flow cytometry and fluorescence microscopy at days 0 and 7. Cell suspensions and conditioned media were analyzed for DNA and secretion of PRG4, respectively. Flow cytometric histograms were deconvolved so that the number of cells in each doubling generation contributing to the final cell population could be
estimated. Chondrocytes were consistently and intensely labeled with PKH26 through 7 cycles of division. At day 7 of culture, >97% of superficial zone cells seeded at low or high density could be distinguished as fluorescent, as could middle zone cells seeded at high density. Retention of cell fluorescence after PKH26 labeling and lack of adverse effects on cell proliferation and synthesis of PRG4 suggest that PKH26 can be useful in determining the fate and function of implanted chondrocytes in vivo, as well as monitoring proliferation in vitro.
2.2 Introduction

Recent studies have examined a number of cell-based tissue-engineering methods for repairing focal cartilage defects [39]. Cells can be delivered into the defect by direct injection [4], under a periosteum flap [9], within a fibrin delivery vehicle [20], or within photopolymerizable hydrogel [13]. Alternatively, cells can be delivered within scaffolds cultured for short (hours to days) [36] or long (weeks to months) [34, 42] durations, as a cartilaginous construct synthesized without a scaffold [18] or as a cartilaginous layer as part of a composite scaffold [5]. Retention of the implanted cells is generally assumed to facilitate cartilage repair and regeneration in all these procedures. However, cell retention can be problematic [4, 36] and delivery by arthroscopic implantation, although having the advantage of being minimally invasive, presents additional challenges for cell retention.

Methods for tracking chondrogenic cells in vitro and in vivo vary in terms of tracer detectability at longer follow-up and versatility in distinguishing cellular organization and cell populations. A variety of probes can be applied. Most fluorescent cytoplasmic dyes (CellTracker™ Green CMFDA, CellTracker™ Orange CMTMR, BCECF-AM; Invitrogen, Carlsbad, CA) are limited to short-term studies of only a few days [19] due to short dye half-lives. In contrast, cell transduction with a reporter gene such as β-galactosidase (lacZ) [3] or Green Fluorescent Protein (GFP) [23] allows for cell tracking as long as the gene is retained and expressed. However, transfection efficiencies of gene delivery into cells, especially in primary isolates or early passages, can be technically difficult and result in transient expression [33];
infection with viral vectors is also challenging and can invoke an immune response [45]. Radioactive probes, such as tritiated thymidine [11] and the thymidine analog bromodeoxyuridine (BrdU) [8], can also be useful for longer-term cell tracking depending on probe lifetimes. Newly synthesized DNA has been detected after as long as 12 weeks \textit{in vivo} [8]. However, determining organization of radiolabeled cells is difficult and quantitative analysis is limited to two-dimensional histological sections by autoradiography [17]. Alternatively, cell populations can be distinguished by intrinsic cell properties. The testis-sex determining region Y gene, \textit{SRY}, which is present in cells originating from male but not female donors or recipients has been used in primary and expanded cells and has been validated in studies up to 12 weeks [36]. This limits cell tracking to allogeneic, gender-specific situations. For all of the above methods, the division history of the implanted cells is difficult to assess. Thus, a more stable fluorescent dye would be useful.

A previously developed stable family of lipophilic fluorescent dyes has been used for tracking transplanted cells. PKH dyes [26] are stably incorporated into the cell membrane and appear to be non-toxic [1, 15, 40]. One of these dyes, PKH26, has been used to track various cell types [22, 32]. Since PKH26 labels the membrane, similar to lipophilic dyes DiI and DiI-derivatives [19, 37] the associated fluorescence signal decreases by half with each cell division cycle [25]. This characteristic has been used to determine the cell number in each doubling generation contributing to a final cell population, thereby assessing proliferation [1, 2, 6, 46].

Tracking chondrocytes with PKH26 could be useful for analysis of cartilage repair \textit{in vivo}. However, the effects of PKH26 on chondrocyte functions should first
be assessed. In repair and in homeostasis, proliferation and secretion of the functional marker molecule proteoglycan 4 (PRG4) [27], encoded by the PRG4 gene [28], and also termed lubricin [29] or Superficial Zone Protein [43], are important. Cartilaginous constructs can be fabricated in a stratified form so that PRG4 secretion is localized at the tissue surface [31]. Thus, the objectives of this study were to establish a cell tracking method using PKH26 for generation analysis of chondrocytes from superficial and deeper zones and to determine the effects of PKH26 on proliferation and secretion of PRG4 protein.

2.3 Materials and Methods

Materials for cartilage explant, chondrocyte isolation, monolayer culture, and biochemical procedures were obtained as described previously [11, 31]. In addition, bovine serum albumin (Fraction V, cell culture tested) and PKH26 labeling kits were obtained from Sigma (St. Louis, MO). Tissue culture treated flasks were from Corning (Corning, NY). Trypsin-EDTA and Hanks’ Balanced Salt Solution (HBSS) were from Invitrogen-Gibco (Grand Island, NY).

Chondrocytes from the superficial and middle layers of immature bovine cartilage were isolated [11, 31]. Articular cartilage slices from the superficial (<0.2 mm depth) and middle (0.4-1.0 mm) layers were harvested from the patellofemoral groove of seven 1-3 week old calf knee joints and digested in medium (Dulbecco’s Modified Eagle Medium [DMEM], 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin,
and 0.25 μg/ml amphotericin B) with 0.2% pronase for 1 hour, and 0.02% collagenase-P for 16 hours.

Superficial and middle zone chondrocytes were expanded in monolayer culture. Cells were plated at low density (10,000 cells/cm²) and grown in tissue culture-treated 175 cm² flasks in complete medium with 10% fetal bovine serum (FBS) and 25 μg/ml ascorbic acid at 37°C in an atmosphere of 5% CO₂/95% air. Each flask received 30 ml medium per 3 days until cells were 80% confluent. Cultures were then serum-starved (incubated in medium with 0.01% BSA) for one day to synchronize cells. Upon release with 0.25% trypsin-1 mM EDTA in HBSS, cells were pelleted, counted, and resuspended in complete medium at about 1 million cells/ml.

To determine the extent of PKH26 retention and the effects of PKH26 labeling on proliferation and secretion of PRG4 by superficial and middle chondrocytes, some cells were labeled with PKH26 (and subsequently designated as S* and M*, respectively).

Some of the released chondrocytes (about 10 million superficial or middle zone cells) were washed once in medium without serum, pelleted, and resuspended in 1 ml of Diluent C (dilution buffer provided in PKH26 cell labeling kit) according to manufacturer’s directions. The cell suspension was mixed with an equal volume of labeling solution containing PKH26 with dilution buffer (final concentration 20 μM) [12]. Chondrocytes were incubated with the labeling solution for 5 min at 25°C with periodic tapping of the tube. The staining reaction was stopped by adding an equal volume of FBS and incubating for 1 min. Cells were washed and pelleted twice thereafter, each time with 5 ml complete medium.
Figure 2.1. Schematic of methods used to evaluate retention of PKH26 by chondrocytes and effects of PKH26 on cell growth and PRG4 secretion. Cells were isolated separately from superficial (S) and middle (M) zones of articular cartilage and allowed to expand in low density monolayer culture. After release, some cells were labeled with PKH26 (indicated by *). Cells were either analyzed immediately or replated in monolayer at low (10,000 cells/cm$^2$) and high (200,000 cells/cm$^2$) densities for an additional 7 days and then released. S (grey) and M (white) zone chondrocytes are shaded differently to emphasize their location in culture.
Different extents of cell division were examined by using different seeding densities. Cells were analyzed immediately or replated in 12 or 24-well dishes at densities of 10,000 or 200,000 cells/cm² and cultured in monolayer for an additional 7 days with 2 ml complete medium per million cells that was changed every 2 days. Cultured cells were then released with trypsin-EDTA (Fig. 2.1).

Qualitative assessments of cellular retention and effects of the PKH26 tag were made by fluorescence microscopy (Nikon Eclipse TE 300, AG Heinze, Irvine, CA) with digital imaging (SPOT RT, Diagnostic Instruments, Burlingame, CA). Phase and fluorescent images were taken of the same field of view of labeled cells, and phase images were taken of untagged cells.

Samples of suspended cells (about 200,000-800,000 cells/ml) were analyzed by flow cytometry. Retention of PKH26 and proliferation by superficial and middle zone chondrocytes were assessed from dye fluorescence intensity profiles. A FACScan flow cytometer with argon ion excitation laser at 488 nm (Becton-Dickinson, Franklin Lakes, NJ; voltage to the fluorescence detector = 395 V) was used to analyze samples. Fluorescence representing PKH26 (peak excitation at 551 nm, peak emission at 567 nm) was collected through a 585/42 nm filter. With these settings, fluorescence intensity values spanned 4 log decades or 1024 channels on a linear scale. For each sample, data representing 10,000 cells were collected as a list-mode file using CellQuest™ software (Becton Dickinson) and then analyzed using FlowJo™ software (Tree Star, Inc., version 5.3, Ashland, OR). Nonviable cells were identified based on propidium iodide staining (data not shown), which had higher signal than PKH26, lower forward scatter intensity, and higher side scatter intensity.
and not analyzed further [1, 2, 6, 46]. A 5% threshold value was used as a gate for discriminating between untagged control cells and tagged cells.

Fluorescence decay associated with PKH26 was calculated assuming that decay obeyed first-order exponential kinetics [44]. The decay was subtracted from fluorescence associated with cells in generation 1. Each subsequent generation thereafter was assigned half the fluorescence of the previous generation, equivalent to a loss of 19.18 channels per generation. Then, a proliferation index (PI) was calculated based on areas of each model-generated distribution contributing to the sample histogram [46]. PI, a measure of proliferation, defined as the ratio of the total number of cells (summation of the Gaussian areas in each generation, $A_k$, up to $k = 10$ generations) to the calculated number of cells initially residing in the parent generation (progenitor cells seeded at day 0):

$$PI = \frac{\sum_{k=1}^{10} A_k}{\sum_{k=1}^{10} \frac{A_k}{2^{k-1}}}$$ (1)

An index of net fluorescence, taking into account proliferation, was calculated as the product of the PI and the mean fluorescence of the sample.

Numbers of cells per generation (cycle of cell division) were determined by fitting day 7 histograms with a Gaussian model of fluorescence distribution, based on day 0 data [46]. A custom program was written in Excel (Microsoft 2002) was written to decompose sample histograms into a sum of distributions representing successive generations by nonlinear least squares fitting. Thus, assuming that fluorescence halved
with each cell division cycle, the number of cells in each doubling generation that contributed to the final cell population was determined.

Cells cultured in monolayer at low and high densities, with and without PKH26, were solubilized with proteinase-K [31]. Portions of the digest were analyzed for DNA using PicoGreen® [35] with calf thymus DNA as the standard. Cell proliferation was quantified as DNA content relative to the number of cells initially seeded, assuming 7.8 pg DNA/cell [30].

Spent media were analyzed for PRG4 secretion by chondrocytes from both zones by indirect ELISA using monoclonal antibody 3A4 (courtesy B. Caterson, Wales, UK) [31]. PRG4 levels were calculated from a standard curve generated from samples containing known amounts of PRG4 based on protein content [43]. Amounts of PRG4 secreted were expressed as pg/(cell•day).

The effects of tagging, seeding density, cell type, day (for some parameters), and interactions were assessed by ANOVA and post-hoc Tukey tests, \( \alpha=0.05 \) (Systat Software 10.2, Richmond, CA). Fluorescence, proliferation, and index of net fluorescence were log-transformed to improve the uniformity of variance among the experimental groups. Data were expressed as mean ± SEM.

### 2.4 Results

Phase and fluorescence images of unlabeled superficial and middle and PKH26-labeled (S*, M*) chondrocytes indicated that PKH26 labeled essentially all cells and that the initial plating density affected the fluorescence intensity present after 7 days of culture. Unlabeled cells plated at low and high densities did not exhibit any
fluorescence, indicative of PKH26 labeling, as expected (Figs. 2.2A-D). By day 7, many of the PKH26-labeled cells that were seeded at low density (Figs. 2.2E,G) appeared dimmer than cells initially seeded at high density (Figs. 2.2F,H) but were still visible. Comparison of phase (Figs. 2.2I-L) and fluorescence (Figs. 2.2E-H) micrographs of PKH26-labeled cells indicated cells retained the dye effectively.
Figure 2.2. Effects of cell growth and seeding density on PKH26 retention by PKH26-labeled S* and M* chondrocytes. Phase (E-H) and fluorescence (I-L) micrographs of S* and M* cells in monolayer culture at day 7 are shown. Micrographs of S* and M* cells are of the same field of view. Unlabeled S (A, B) and M (C, D) cells are also shown. Cells seeded at low (10,000 cells/cm²; A, C, E, G, I, K) and high (200,000 cells/cm²; B, D, F, H, J, L) seeding densities are indicated. Bar = 100 μm.
A high proportion of labeled cells retained PKH26-associated fluorescence after 7 days of culture, as analyzed by flow cytometry. Both superficial and middle zone cells were labeled brightly and with a high efficiency at day 0 (98.9±0.3% and 97.9±1.1%, respectively, Fig. 2.3). By day 7, 97.3±0.4% of superficial cells seeded at both densities were fluorescently tagged, as were middle zone cells seeded at high density (Fig. 2.3). Middle zone cells seeded at low density proliferated more, so a lower percentage (87.9±5.3%) retained sufficient PKH26 to be identified after 7 days.

Generation analysis of flow cytometric histograms (Fig. 2.3) allowed discrimination of cells up through at least 7 successive generations (based on the location of a 5% gate, identifying unlabeled cells). A representative day 0 histogram shows the fluorescence distribution of cells immediately after labeling with PKH26 (Fig. 2.4A) with the calculated distribution of cells per generation (Fig. 2.4B). As expected, the majority (93% for the sample in Fig. 2.4B) of the cells were computed to be in the first generation. After 7 days, some cells had diminished intensity. A shift in the fluorescence distribution of cells by day 7 was observed (Fig. 2.4C) with cells seeded at low density shifted the most. Compared to the day 0 distribution (Fig. 2.4B), the distribution of cells per generation for these cells (Fig. 2.4D) was noticeably wider, with cells distributed among many generations; the largest percentage (32%) of cells was in generation 5.
Figure 2.3. Effects of seeding density and time in monolayer culture on PKH26 retention by (A) S* and (B) M* chondrocytes. Cell cycles were synchronized by 1 day of serum starvation prior to release from monolayer expansion culture. Cell suspensions were analyzed for fluorescence by flow cytometry. Percentage of cells retaining PKH26 were calculated for low and high cell densities was calculated based on the position of gate for unlabeled cells (dotted line). (n=6-7).
Figure 2.4. Cell suspensions were analyzed for fluorescence by flow cytometry. Numbers of cells per generation (cycle of cell division) were determined by fitting day 7 histograms from a model of Gaussian fluorescence distribution, based on day 0 data. (A) A representative sample of labeled cells on day 0. (B) Accompanying distribution of cells per generation for the day 0 sample shown in (A). (C) A representative sample of $S$ cells initially seeded at 10,000 cells/cm$^2$ at day 7 with respective generation numbers indicated. (D) The accompanying distribution of cells per generation for the sample shown in (C).
According to generation analysis, cell proliferation was affected by seeding density (p<0.001) but not chondrocyte origin (p=0.33), although a significant interaction of the two factors (p<0.05) was found (Fig. 2.5). At day 0, 80.2±2.3% of the cells in each population were calculated to be in generation 1 (Figs. 2.5A,D). As expected, at day 7, more than half of the cells seeded at high density resided in generation 4 or lower, compared to cells seeded at low density, which had a high percentage of cells shifted into higher generations (Figs. 2.5B,C). While seeding density had an overall effect, the median generation for superficial cells seeded at low density was 4.9±0.3, significantly higher (p<0.001) than the median generation of 2.3±0.4 at high cell density (Figs. 2.5E,F). No significant difference was found between the median generation of middle zone cells seeded at low or high cell densities (p=0.17).

The PI calculated from the generation model for S* and M* chondrocytes after 7 days of culture was significantly affected by cell type (p<0.05) and density (p<0.001) with a significant interaction of the two factors (p<0.05). The PI was greater (p<0.001) in superficial cultures initiated at low density (7.2±0.7-fold) than those initiated at high density (2.7±0.7-fold). Middle cultures seeded at low and high densities proliferated at approximately the same rates (p=0.6) (7.9±0.1-fold, low; 5.9±0.9-fold, high) (Fig. 2.6A). By day 7, the number of middle zone cells present at high density was 3.1±0.9 times more than superficial cells at high density (p<0.05). At low density, the PI was not different (p=1.00) between cell types (Fig. 2.6A).
Figure 2.5. Effects of chondrocyte origin and seeding density on proliferation. Chondrocytes were isolated from the S and M layers of articular cartilage, plated at low or high cell densities, cultured for 7 days, and analyzed for the percentage of cells in different generations. (A) Distribution of S (♦) and M (◊) chondrocytes per generation at day 0. At day 7, S (B) and M (C) chondrocytes plated at low (■) and high (Δ) densities were analyzed for the percentage of cells in different generations. Median generations were also determined (D-F). Data are expressed as mean±SEM (n=6-7). P = 0.001 (●).
The mean fluorescence intensity of PKH26-labeled cells after 7 days was significantly affected by density (p<0.001) and cell type (p<0.001) with a trend towards an interactive effect (p=0.08; Fig. 2.6B). Intensity of cells seeded at low density decreased 12.9±2.6-fold after 7 days (p<0.001) while those seeded at high density decreased significantly less, 6.5±1.0-fold (p<0.001). Mean intensity of superficial cells was 2.5±0.4-fold (p<0.001) higher than middle zone cells after 7 days. Intensity of cells seeded at high density was 2.5±0.3-fold (p<0.05) higher than cells seeded at low density by day 7.

The index of net fluorescence was significantly affected by cell type (p<0.001) and density (p<0.001), with a significant interactive effect (p<0.05, Fig. 2.6C). By day 7, superficial chondrocytes seeded at high density had a 2.4±0.2-fold loss in the index (p<0.001) while middle zone cells seeded at low density had a 2.8±0.1-fold loss (p<0.001). At low density, superficial cells had a 2.5±0.4-fold greater loss in the index compared to middle zone cells at low density (p<0.001). Cells at high density also had a higher loss in the index than cells seeded at low density (2.1±0.5-fold superficial; 2.7±0.3-fold middle) (p<0.05). Thus, when proliferation was considered, the net fluorescence (associated with the entire population of cells in a given culture condition) was altered 2.0±0.2-fold, on average, during in vitro culture.

Cell proliferation over the 7 days remained unaffected by PKH26 labeling (p=0.33), but was significantly affected by density (p<0.001) with a trend for an effect of cell type on relative proliferation (p=0.08). No significant interactions were found (p=0.12-0.98). During the 7 days, proliferation was 1.3±0.2-fold greater by middle zone than superficial cultures (p=0.08) and greater (p<0.001) in cultures initiated at
low (9.7±1.0-fold, superficial; 13.2±1.2-fold, middle) than those initiated at high density (2.0±0.1-fold, superficial; 2.2±0.2-fold, middle) density (Fig. 2.7A).

PRG4 secretion by superficial cells was significantly greater than that by middle zone cells (p<0.001) and significantly affected by density (p<0.001) but not by PKH26 tagging (p=0.81) (Fig. 2.7B). A significant interactive effect occurred between cell type and density (p<0.001). Superficial chondrocytes at low density secreted 1.1±0.3 pg/(cell•day) by day 7 in culture, significantly lower than the 9.0±1.6 pg/(cell•day) secreted by superficial cells seeded at high density (p<0.001). Middle zone cells secreted PRG4 at a much lower rate, averaging 0.3±0.1 pg/(cell•day), than superficial cells at high density (p<0.001), but this rate was not significantly different from superficial cells seeded at low density (p=0.76-0.92).
Figure 2.6. Effects of chondrocyte origin and seeding density on retention of PKH26 in vitro. (A) Proliferation index of tagged cells at day 7, normalized to proliferation index of day 0 samples, as determined from generation model. (B) Mean fluorescence of cells at day 0 and day 7. (C) Index of net fluorescence was calculated as product of mean fluorescence and proliferation index. Data are expressed as mean±SEM (n=6-7). $P < 0.001 (\bullet), P < 0.05 (\circ).$
Figure 2.7. Effects of cell type, tagging, and cell density on (A) relative proliferation and (B) PRG4 secretion. Relative proliferation was determined as the number of cells present on day 7 (PicoGreen® assay for DNA) normalized to number of cells seeded. Spent medium collected on day 6 of culture was analyzed for PRG4 secretion by indirect ELISA using monoclonal antibody 3A4 (B. Caterson) and normalized to number of cells present on day 7 of monolayer culture. Data are expressed as mean±SEM (n=4-7).
2.5 Discussion

These results establish methods for using PKH26 in studies of chondrocyte growth without adverse effects on chondrocyte function, specifically, cellular proliferation and secretion of PRG4. Fluorescence microscopy (Fig. 2.2) and analysis of cell suspensions by flow cytometry (Fig. 2.3) both indicated strong retention of PKH26 by superficial and middle zone chondrocytes after 7 days in monolayer culture, whether cells were induced to proliferate at a low rate (seeding at high density, 200,000 cells/cm²) or at a high rate (seeding at a low density, 10,000 cells/cm²). By day 7, fluorescence in 91% of these cells was still detectable by flow cytometry, while the net fluorescence was somewhat decreased for both cell types and seeding densities (Fig. 2.6). The possibility of tracking PKH26-labeled cells through several generations was established from flow cytometric data (Figs. 2.4 and 2.5). PKH26 labeling did not affect proliferation rate or secretion of PRG4 (Fig. 2.7).

In the future, longer culture durations and other cell sources should be analyzed, since such cell cultures may exhibit different responses. In addition, the effects of PKH26 on a variety of cell functions should be delineated. While proliferation is an important feature of isolated chondrocytes for tissue engineering, and PRG4 expression is characteristic of superficial cells, cell fate processes such as (re)differentiation are also important and may be affected by PKH26.

PKH26 effectively labeled several cell types [21, 32, 40] in studies for tracking of lymphocytes during 60-day homing studies in rats [14] and germ cells over 12 weeks after transplantation in goats [24]. PKH26 was also used to track the fate of 4-cell stage bovine embryos during blastocyst formation [38]. Localization of PKH26-
labeled cells is useful since the dye does not passively transfer to unlabeled cells, such as after injection of labeled cells in vivo [14] or in co-cultures of labeled and unlabeled cells in vitro [2, 14]. PKH26 labeling would seem applicable in cartilage repair studies since, superficial and middle zone chondrocytes isolated from cartilage of 4-6 month old Yucatan mini-pigs have also been effectively labeled [10], and PKH26-labeled chondrocytes injected in an autologous chondrocyte implantation procedure in goats indicated retention of fluorescently labeled cells after 14 weeks in vivo [12].

The utility of PKH26 for assessing cell proliferation follows from its bright fluorescence and utility for cell tracking. Previous studies developed flow cytometric methods for assessing proliferation with PKH26 in cell types other than those that are skeletally differentiated; the reduction of fluorescence intensity with chondrocyte division (Fig. 2.3) generally agrees with such studies [1, 2, 6, 7, 16, 46]. The diminution of fluorescence is consistent with dilution during proliferation since PKH26-associated fluorescence does not decrease significantly in non-dividing cells [2, 7].

PKH26 does not adversely affecting intrinsic cellular functions including proliferation (Fig. 2.7A), consistent with other studies [6, 7, 14]. Effects on proliferation were similar, whether determined with PKH26 (by generation analysis) or by PicoGreen® assay (for DNA content). Differences in the extent of proliferation, for example, with middle zone chondrocytes seeded at high density having a higher proliferation index by generation analysis flow cytometry than by DNA assay, may be due to a number of factors. Only viable cells were considered during the flow cytometry analysis, whereas both viable and non-viable cells were quantified by DNA
content analysis. Also, photobleaching, expected with light exposure of cultures during medium changes, would lead to overestimation of proliferation by generation analysis.

Ideally, supravital dyes should not have harmful effects on other cellular functions, such as PRG4 secretion by chondrocytes. The inertness of PKH26 labeling on PRG4 secretion by chondrocytes is consistent with its lack of adverse effects on cell viability [1, 15, 40] and growth [6, 7, 14] and embryonic development [38]. In the present study, PRG4 secretion levels were comparable to those found previously for superficial and middle zone chondrocytes in monolayer culture [41].

Tracking chondrocytes labeled with PKH26 could have widespread applications for evaluating implanted cells in articular cartilage repair in vivo, especially in biopsies or terminal analyses in animal models. Tracking culture-expanded chondrocytes could help determine cell retention at the repair site and redifferentiation. Ideally, cells for implantation would be labeled as close as possible to the implantation time to minimize the fluorescence decrease due to division prior to implantation. Prior to applying PKH26 labeling to track chondrocytes quantitatively, the number of cell divisions that PKH26-labeled chondrocytes could undergo and still be detected had to be determined. Our results suggest that for constructs implanted in vivo with PKH26-labeled cells, any recovered cells that were PKH26 positive would have undergone less than 7 cycles of cell division. Conversely, those that are PKH26-negative would either be from the host or be implanted cells that have proliferated extensively.
Several lines of reasoning suggest that PKH26 will be useful even in the presence of a local inflammatory joint reaction. The structure of PKH26 [19] makes it unlikely to be cleaved by proteases, including collagenases, or other mammalian enzymes (personal communication, Molecular Probes). Also, when PKH26-labeled cells are released with cell dissociation enzyme preparations (e.g., based on trypsin), a high proportion (99%) are clearly labeled. The finding that PKH26 labeling did not adversely affect chondrocyte proliferation and PRG4 secretion is significant for future in vivo cell tracking studies aimed at gaining insight into mechanisms underlying articular cartilage repair.

2.6 Acknowledgments

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2.7 References


CHAPTER 3

TRACKING CHONDROCYTES AND ASSESSING THEIR PROLIFERATION WITH CFSE: EFFECTS ON CELL FUNCTION AND SECRETION OF PROTEOGLYCAN 4 (PRG4)

3.1 Abstract

Objective: To assess the utility of CFSE for tracking chondrocytes from superficial and middle zones and their proliferation, and determine the effects of CFSE on chondrocyte functions, in particular, proliferation, PRG4 secretion (Proteoglycan 4), and matrix (glycosaminoglycan (GAG)) content.

Design: CFSE-labeled and unlabeled superficial and middle zone chondrocytes were plated in either low or high density monolayer culture and analyzed for retention of CFSE by flow cytometry and fluorescence microscopy at days 0 and 7. Cell suspensions were analyzed for DNA and GAG and conditioned media for secretion of PRG4. Flow cytometric histograms were deconvolved so that the number of cells in each doubling generation contributing to the final cell population could be determined.

Results: Chondrocytes were consistently and intensely labeled with CFSE through 10 cycles of division. At day 7 of culture, >97% of superficial zone cells seeded at low or high density could be distinguished as fluorescent, as could middle zone cells seeded...
at high density. Proliferation, matrix content, and synthesis of PRG4 were unaffected by cell labeling.

**Conclusions:** Retention of cell fluorescence and minimal adverse effects on cell proliferation, matrix content, and synthesis of PRG4 suggest that CFSE can be useful in determining the fate and function of implanted chondrocytes *in vivo*, and monitoring proliferation *in vitro*. 

3.2 Introduction

Several cell-based tissue-engineering methods have been examined for repair of focal cartilage defects [34]. Cells can be delivered into the defect by direct injection [4], under a periosteum flap [7], within a fibrin delivery vehicle [20], or within photopolymerizable hydrogel [14]. Alternatively, cells can be delivered within scaffolds cultured for short (hours to days) [32] or long (weeks to months) [29, 37] times, as a cartilaginous construct synthesized without a scaffold [17] or as a cartilaginous layer as part of a composite scaffold [5]. Retention of the implanted cells is generally assumed to facilitate cartilage repair and regeneration in all these procedures. However, cell retention can be problematic [4, 32] and delivery by arthroscopic implantation, although having the advantage of being minimally invasive, presents additional challenges for cell retention.

Tracking methods using fluorescent dyes have been implemented in order to allow localization of individual cells. PKH dyes [21] are stably incorporated into the cell membrane, allowing for proliferation assessment [1, 2, 6, 11, 43], and appear to be non-toxic [1, 11, 16, 35]. PKH26 has previously been applied to track chondrocytes in vitro [11] and also chondrocytes implanted in vivo either by autologous chondrocyte transplantation [12] or implant of stratified cartilaginous tissues containing PKH26-labeled subpopulations [10]. Most fluorescent cytoplasmic dyes (CellTracker™ Green CMFDA, CellTracker™ Orange CMTMR, BCECF-AM), for tracking cells and determining cellular organization are limited to short-term studies lasting only a few days [19] due to dye dilution with cellular division. A longer-lasting cytoplasmic dye is
carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) [33] which passively diffuses into cells and is highly fluorescent and well-retained once its acetate groups are cleaved by intracellular esterases [18, 33]. CFSE has been used to track various cell types [3, 27], and similar to lipophilic dyes, can be applied to track generations of cells since the associated fluorescence signal decreases by half with each cell division cycle. CFSE does not appear to have adverse effects on cell function and cell viability [42] indicating that it could be useful for tracking chondrocytes in cell-laden tissues implanted in vivo.

Prior to application of CFSE cartilage repair analyses in vivo, the effects of CFSE on chondrocyte function must first be assessed. In repair and in homeostasis, proliferation, matrix content of cells, and secretion of the functional marker molecule Lubricin [24] or Superficial Zone Protein (SZP) [38], encoded by the gene PRG4 [23], and also termed Proteoglycan 4 (PRG4) [22] are important. Thus, the objectives of this study were to establish a cell tracking method using CFSE for generation analysis of chondrocytes from superficial and deeper zones and to determine the effects of CFSE on proliferation, matrix content of cells, and secretion of PRG4 protein.

### 3.3 Materials and Methods

Materials for cartilage explant, chondrocyte isolation, monolayer culture, and biochemical procedures were obtained as described previously [11]. In addition, CFSE labeling kit was from Invitrogen/Molecular Probes (Carlsbad, CA).

Chondrocytes from the superficial (S, <0.2 mm) and middle (M, 0.4-1.0 mm) layers of immature bovine cartilage were isolated (n=6, 1-3 week old calf knee joints)
Chondrocytes from each animal were expanded in low density (10,000 cells/cm²) monolayer culture in medium (Dulbecco’s Modified Eagle Medium [DMEM], 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B). Cells were serum-starved for one day to synchronize cultures prior to release with 0.25% trypsin-1 mM EDTA. Upon release with 0.25% trypsin-1 mM EDTA in Hanks’ Balanced Salt Solution (HBSS), cells were pelleted, counted, and resuspended in complete medium at about 1 million cells/mL.

To determine the extent of CFSE retention and the effects of CFSE labeling on proliferation, GAG content, and secretion of PRG4 by S and M chondrocytes, some cells were labeled with CFSE (and subsequently designated as S^ or M^). Some of the released chondrocytes (about 10 million S or M cells) were washed once in HBSS and then resuspended in 5 ml of pre-warmed labeling solution containing CFSE (final concentration 20 μM in phosphate buffered saline (PBS), [18]). Chondrocytes in suspension were incubated with the labeling solution for 15 min at 37°C. Cells were pelleted by centrifugation and resuspended in medium containing 10% FBS. Cells were washed once thereafter with complete medium.

Different extents of cell division were examined by using different seeding densities. Cells were analyzed immediately or replated in 6, 12, or 24-well dishes at densities of 10,000 or 200,000 cells/cm² and cultured in monolayer for an additional 1 or 7 days with 2 ml complete medium per million cells that was changed every 2 days. Cultured cells were then released with trypsin-EDTA (Fig. 3.1).
Figure 3.1. Schematic of methods used to evaluate CFSE retention by chondrocytes and effects of CFSE on cell growth, GAG content, and PRG4 secretion. Cells were isolated separately from superficial and middle zones of articular cartilage and allowed to expand in low density monolayer culture. After release, some cells were labeled with CFSE (indicated by ^). Cells were either analyzed immediately or replated in monolayer at low (10,000 cells/cm²) and high (200,000 cells/cm²) densities for an additional 7 days and then released. Superficial (grey) and middle (white) zone chondrocytes are shaded differently to emphasize their location in culture.
Qualitative assessments of cellular retention and effects of the CFSE tag were made by fluorescence microscopy (Nikon Eclipse TE 300, AG Heinze, Irvine, CA) with SPOT RT digital imaging (Diagnostic Instruments, Burlingame, CA). Phase and fluorescent images were taken of the same field of view of labeled cells, and phase images were taken of untagged cells.

Samples of suspended cells (about 200,000-800,000 cells/ml) were analyzed by flow cytometry as previously described [11]. Fluorescence representing CFSE (peak excitation at 492 nm, peak emission at 517 nm) was collected through a 530/30 nm filter. For each sample, data representing 10,000 cells were collected as a list-mode file using CellQuest™ software (Becton Dickinson) and then analyzed using FlowJo™ software (Tree Star, Inc., version 6.4.1, Ashland, OR). A 5% threshold value was used as a gate for discriminating between untagged control cells and tagged cells.

Fluorescence decay of associated with CFSE was calculated assuming that decay obeyed first-order exponential kinetics [39]. The decay was subtracted from fluorescence associated with cells in generation 1. Each subsequent generation thereafter was assigned half the fluorescence of the previous generation, equivalent to a loss of 19.18 channels per generation. Then, a proliferation index (PI) was calculated based on areas of each model-generated distribution contributing to the sample histogram [11, 43]. PI, a measure of proliferation, was the ratio of the total number of cells (summation of the Gaussian areas in each generation, $A_k$, up to $k = 10$ generations) to the calculated number of cells initially residing in the parent generation (progenitor cells seeded at day 0):
\[
PI = \frac{\sum_{k=1}^{10} A_k}{\sum_{k=1}^{10} \frac{A_k}{2^k}}
\]  

(1)

An index of net fluorescence, taking into account proliferation, was calculated as the product of the proliferation index (PI) and the mean fluorescence of the sample. Numbers of cells per generation (cycle of cell division) were determined by fitting day 1 and day 7 histograms with a Gaussian model of fluorescence distribution, based on day 0 data [11]. A previously written custom program (Microsoft Excel 2002, Bellevue, WA) was used to decompose sample histograms into a sum of distributions representing successive generations by nonlinear least squares fitting [11]. Thus, assuming that fluorescence halved with each cell division cycle, the number of cells in each doubling generation that contributed to the final cell population was determined.

Cells cultured in monolayer at low and high densities, with and without CFSE, were solubilized with proteinase-K [26]. Portions of the digest were analyzed for DNA using Hoechst 33258 [25], with calf thymus DNA as the standard, or GAG by DMMB [15].

Spent media were analyzed for PRG4 secretion by chondrocytes from both zones by indirect ELISA using monoclonal antibody 3A4 (courtesy B. Caterson, Wales, UK) [26]. PRG4 levels were calculated from a standard curve generated from samples containing known amounts of PRG4 based on protein content [38]. Amounts of PRG4 secreted were expressed as pg/(cell•day).
The effects of tagging, seeding density, cell type, day, and interactions were assessed by ANOVA and post-hoc Tukey tests, $\alpha=0.05$ (Systat Software 10.2, Richmond, CA). Data were expressed as mean ± SEM.

### 3.4 Results

Phase and fluorescence images of unlabeled superficial and middle and CFSE-labeled ($S^\wedge$, $M^\wedge$) chondrocytes indicated that CFSE labeled essentially all cells and that the initial plating density affected the fluorescence intensity present after 7 days of culture. Unlabeled cells plated at low and high densities did not exhibit any fluorescence, indicative of CFSE labeling, as expected (Fig. 3.2A, D, G, J, M, P, S, V). On day 1, CFSE-labeled cells at low and high density were labeled brightly (Fig. 3.2C, I, O, U). By day 7, many of the CFSE-labeled cells that were seeded at low density (Fig. 3.2F, R) appeared dimmer than cells initially seeded at high density (Fig. 3.2L, X) but were still visible. Comparison of phase (Fig. 3.2B, D, H, J, N, P, T, V) and fluorescence (Fig. 3.2C, F, I, L, O, R, U, X) micrographs of CFSE-labeled cells indicated cells retained the dye effectively.
**Figure 3.2.** Effects of cell growth and seeding density on CFSE retention by CFSE-labeled S^ and M^ chondrocytes. Phase (B, E, H, K, N, Q, T, W) and fluorescence (C, F, I, L, O, R, U, X) micrographs of S^ and M^ cells in monolayer culture at day 1 (B, C, H, I, N, O, T, U) and day 7 (E, F, K, L, Q, R, W, X) are shown. Micrographs of S^ and M^ cells are of the same field of view. Unlabeled S (A, D, G, J) and M (M, P, S, V) cells at day 1 (A, G, M, S) and day 7 (D, J, P, V) are also shown. Cells seeded at low (10,000 cells/cm²; A-F, M-R) and high (200,000 cells/cm²; G-L, S-X) seeding densities are indicated. Bar = 100 μm.
A high proportion of labeled cells retained CFSE-associated fluorescence after 7 days of culture, as analyzed by flow cytometry. Both superficial and middle zone cells were labeled brightly and with a high efficiency at day 1 at both densities (98.8±0.3% and 98.9±0.6%, respectively, Fig. 3.3AB). By day 7, 93.2±4.4% of superficial cells seeded at low density and 99.2±0.3% of superficial cells seeded at high density were fluorescently tagged, as were middle zone cells seeded at high density (98.4±0.6%) (Fig. 3.3B). Middle zone cells seeded at low density proliferated more, so a lower percentage (89.4±5.3%) retained sufficient CFSE to be identified after 7 days.

According to generation analysis, cell proliferation was affected by seeding density (p<0.01) and time in culture (p<0.001) but not chondrocyte origin (p=0.2). There was a significant interaction between seeding density and time in culture (p=0.05) (Fig. 3.4). At day 1, most of the cells in each population were calculated to be in generation 1 (Fig. 3.4A-D). At day 7, more than half of the cells seeded at low density resided in generation 6 or higher, compared to cells seeded at high density, which had a high percentage of cells still in lower generations (Fig. 3.4E-H). While seeding density had an overall effect, the median generation for cells initially seeded at low density was 1.9±0.2 on day 1 and significantly increased (p<0.001) to 8.1±0.5 by day 7. Similarly, the median generation of cells initially seeded at high density as 1.5±0.2 on day 1 and significantly increased (p<0.01) to 1.8±0.5 by day 7.
Figure 3.3. Effects of seeding density and time in monolayer culture on CFSE retention by (A) S\(^+\) and (B) M\(^+\) chondrocytes. Cell cycles were synchronized by 1 day of serum starvation prior to release from monolayer expansion culture. Cell suspensions were analyzed for fluorescence by flow cytometry on days 1 and 7. Percentage of cells retaining CFSE were calculated for low (blue) and high (green) cell densities was calculated based on the position of gate for unlabeled cells (dotted line). (n=3).
According to generation analysis, cell proliferation was affected by seeding density (p<0.01) and time in culture (p<0.001) but not chondrocyte origin (p=0.2). There was a significant interaction between seeding density and time in culture (p=0.05) (Fig. 3.4). At day 1, most of the cells in each population were calculated to be in generation 1 (Fig. 3.4A-D). At day 7, more than half of the cells seeded at low density resided in generation 6 or higher, compared to cells seeded at high density, which had a high percentage of cells still in lower generations (Fig. 3.4E-H). While seeding density had an overall effect, the median generation for cells initially seeded at low density was 1.9±0.2 on day 1 and significantly increased (p<0.001) to 8.1±0.5 by day 7. Similarly, the median generation of cells initially seeded at high density as 1.5±0.2 on day 1 and significantly increased (p<0.01) to 1.8±0.5 by day 7.

The PI calculated from the generation model for S^ and M^ chondrocytes after 7 days of culture was significantly affected by time in culture (p<0.001) and density (p<0.001) with a significant interaction of the two factors (p<0.05) (Fig. 3.5A). The PI was greater for cells at day 7 (49.9±9.9) compared to cells at day 1 (1.8±0.2) (p<0.001). In addition, the PI was higher for cells seeded at low density (41.4±12.4) by day 7, compared to cells seeded at high density (17.2±5.5) (p<0.01). The PIs of superficial and middle cells were similar (p=0.7).
Figure 3.4 Effects of chondrocyte origin and seeding density on proliferation. Chondrocytes were isolated from the S and M layers of articular cartilage, plated at low or high cell densities, cultured for 7 days, and analyzed for the percentage of cells in different generations. (A, E) Percentage of S and (C, G) M chondrocytes per generation at day 1 (A, C) and day 7 (E, G) at low (■) and high (△) cell densities. Median generations were also determined (B, D, F, H). Data are expressed as mean±SEM (n=3).
The mean fluorescence intensity of CFSE-labeled cells after 7 days was significantly affected by time in culture (p<0.001) but not by cell type (p=0.8), density (p=0.6), or interactions of the aforementioned factors (p>0.6; Fig. 3.5B). Intensity of cells at day 1 was 69.2±13.7-fold higher than cells at day 7. Mean fluorescence intensity of superficial and middle cells were similar on day 1 of culture (p=0.8). Similarly, mean fluorescence intensity of cells seeded at low density was similar to that at high density (p=0.6).

The index of net fluorescence was significantly affected by time in culture (p<0.05) with a trend towards an interactive effect of cell type and time in culture (p=0.09) (Fig. 3.5C). By day 7, superficial chondrocytes had a 1.5±0.2-fold loss in the index while middle cells had a 4.0±1.1-fold loss (p=0.09). Loss in the index was not affected by cell type (p=0.8) or density (p=0.8). Thus, when proliferation was considered, the net fluorescence (associated with the entire population of cells in a given culture condition) was altered 2.9±0.7-fold, on average, during in vitro culture.

Cell proliferation over the 7 days remained unaffected by CFSE labeling (p=0.8), but was significantly affected by density (p<0.05) with a trend for an effect of cell type (p=0.07) (Fig. 3.6A). No significant interactions were found (p>0.6) (Fig. 3.6A). During the 7 days, proliferation was 2.4±0.5-fold greater by middle zone than superficial cultures (p=0.07) and greater (p<0.05) in cultures initiated at low (12.3±2.8-fold, superficial; 16.0±2.4-fold, middle) than those initiated at high density (3.3±0.8-fold, superficial; 10.2±3.3-fold, middle) density (Fig. 3.6A).
Figure 3.5. Effects of chondrocyte origin and seeding density on retention of CFSE in vitro. (A) Proliferation index of tagged cells at day 7, normalized to proliferation index of day 0 samples, as determined from generation model. (B) Mean fluorescence of cells at day 0 and day 7. (C) Index of net fluorescence was calculated as product of mean fluorescence and proliferation index. Data are expressed as mean±SEM (n=3).
PRG4 secretion by superficial cells was significantly greater than that by middle zone cells (p<0.01) with a trend towards an effect of density (p=0.1) and no significant effect of CFSE tagging (p=0.7) (Fig. 3.6B). A trend towards a significant interactive effect of cell type and density was noted (p=0.07) but no other significant interactions were found (p>0.7). Superficial chondrocytes at low density secreted 3.2±1.6 pg/(cell•day) by day 7 in culture, lower than the 10.3±4.1 pg/(cell•day) secreted by superficial cells seeded at high density (p=0.1). Middle zone cells secreted PRG4 at a much lower rate, 1.1±0.8 pg/(cell•day) and 0.2±0.1 pg/(cell•day), at low and high densities, respectively.

By day 7 in culture, GAG content of cell layers (normalized to DNA of cells adherent on day 1) seeded at low density was significantly higher (3.0-fold) than cell layers seeded at high density (p<0.05) (Fig. 7). GAG/DNA in the cell digest was not affected by CFSE label (p=0.1) or cell type (p=0.2). Interactions among the factors of cell type, label, and seeding density also were not statistically significant (p=0.1-0.5) GAG secreted into culture media (normalized to DNA of cells adherent on day 1) was unaffected cell type (p=0.4) and label (p=0.3). GAG/DNA in the conditioned media was affected by cell density (p<0.05). Interactions were not statistically significant (p=0.4-0.8).
Figure 3.6. Effects of cell type, tagging, and cell density on (A) relative proliferation and (B) PRG4 secretion. Relative proliferation was determined as the number of cells present on day 7 (PicoGreen® assay for DNA) normalized to number of cells seeded. Spent medium collected on day 6 of culture was analyzed for PRG4 secretion by indirect ELISA using monoclonal antibody 3A4 and normalized to number of cells present on day 7 of monolayer culture. Data are expressed as mean±SEM (n=3).
Figure 3.7. Effects of cell type, tagging, and cell density on GAG secretion. GAG content in cell digest (■) or conditioned media (□) was assessed on day 7 by DMMB assay. Data are expressed as mean±SEM (n=2-3).
3.5 Discussion

These results establish methods for using CFSE in studies of chondrocyte growth without adverse effects on chondrocyte function, specifically, cellular proliferation and secretion of PRG4. Fluorescence microscopy (Fig. 3.2) and analysis of cell suspensions by flow cytometry (Fig. 3.3) both indicated strong retention of CFSE by superficial and middle zone chondrocytes after 7 days in monolayer culture, whether cells were induced to proliferate at a low rate (seeding at high density, 200,000 cells/cm$^2$) or at a high rate (seeding at a low density, 10,000 cells/cm$^2$). By day 7, fluorescence in 97% of these cells was still detectable by flow cytometry, while the net fluorescence was somewhat decreased for both cell types and seeding densities (Fig. 3.3). The possibility of tracking CFSE-labeled cells through several generations was established from flow cytometric data (Figs. 3.4, 3.5). CFSE labeling did not affect proliferation rate or secretion of PRG4 (Fig. 3.6) and had minimal effects on GAG content of middle cells.

In the future, longer culture durations and other cell sources should be analyzed, since such cell cultures may exhibit different responses. In addition, the effects of CFSE on a variety of cell functions should be delineated. While proliferation is an important feature of isolated chondrocytes for tissue engineering, and PRG4 expression is characteristic of superficial cells, cell fate processes such as (re)differentiation are also important and may be affected by CFSE.

CFSE effectively labeled several cell types in studies for analyzing neural crest migration [8], separation of myogenic and fibroblastic cells [31], homing of
hematopoietic stem cells [13], and proliferation of airway smooth muscle cells [40]. CFSE was also used to track the fate of human intervertebral disc cells in vitro [18]. Localization of CFSE-labeled cells is useful since the dye does not passively transfer to unlabeled cells [41] and has been shown to not transfer between labeled and unlabeled cells in co-culture experiments in vitro [31]. CFSE labeling would seem applicable in cartilage repair studies since, superficial and middle zone chondrocytes isolated from cartilage of 4-6 month old Yucatan mini-pigs have also been effectively labeled [10], and could be used in conjunction with PKH26, another fluorescent previously described for tissue engineering applications [10, 11].

The utility of CFSE for assessing cell proliferation follows from its bright fluorescence and utility for cell tracking. Previous studies developed flow cytometric methods for assessing proliferation with CFSE in cell types other than those that are skeletally differentiated; the reduction of fluorescence intensity with chondrocyte division (Fig. 3.3) generally agrees with such studies [28, 33]. CFSE dye has recently been applied to quantify lymphocyte kinetics in vivo, specifically to estimate the rate of proliferation and death of lymphocytes in situ [3].

CFSE does not adversely affect intrinsic cellular functions including proliferation (Fig. 3.6A), consistent with other studies [27, 28]. Effects on proliferation were similar, whether determined with CFSE (by generation analysis) or by PicoGreen® assay (for DNA content). Differences in the extent of proliferation, for example, with middle zone chondrocytes seeded at high density having a higher proliferation index by generation analysis flow cytometry than by DNA assay, may be due to a number of factors. Only viable cells were considered during the flow
cytometry analysis, whereas both viable and non-viable cells were quantified by DNA content analysis. Also, photobleaching, expected with light exposure of cultures during medium changes, would lead to overestimation of proliferation by generation analysis. Similar differences were noted in determining the extent of proliferation with PKH26 dye [11].

Supravital dyes should not have harmful effects on other cellular functions, such as PRG4 secretion by and matrix content of chondrocytes. The inertness of CFSE labeling on PRG4 secretion by chondrocytes is consistent with its lack of adverse effects on cell viability [27, 42] and growth [40]. In the present study, PRG4 secretion levels were comparable to those found previously for superficial and middle zone chondrocytes in monolayer culture [11, 36]. While CFSE labeling did not have a statistically significant effect on matrix content, the matrix content of labeled middle chondrocytes appeared to be lower than unlabeled middle cells. In the future, lower concentrations of CFSE dye could be used and/or applied in conjunction with growth factor stimulation, such as bone morphogenetic protein-7 (BMP-7, or osteogenic protein-1, OP-1) which has been shown to increased matrix deposition in cartilaginous tissues generated in vitro [9, 30].

Tracking chondrocytes labeled with CFSE could have widespread applications for evaluating implanted cells in articular cartilage repair in vivo, especially in biopsies or terminal analyses in animal models. Tracking culture-expanded chondrocytes could help determine cell retention at the repair site and redifferentiation. Ideally, cells for implantation would be labeled as close as possible to the implantation time to minimize the fluorescence decrease due to division prior to
implantation. Prior to applying CFSE labeling to track chondrocytes quantitatively, the number of cell divisions that CFSE-labeled chondrocytes could undergo and still be detected had to be determined. Our results suggest that for constructs implanted in vivo with CFSE-labeled cells, any recovered cells that were CFSE positive would have undergone less than 10 cycles of cell division. Conversely, those that are CFSE-negative would either be from the host or be implanted cells that have proliferated extensively. The finding that CFSE labeling did not adversely affect chondrocyte proliferation and PRG4 secretion is significant for future in vivo cell tracking studies aimed at gaining insight into mechanisms underlying articular cartilage repair.

3.6 Acknowledgments

This chapter will be submitted, in full, for publication to Osteoarthritis & Cartilage. The dissertation author is the primary investigator and thanks co-authors, Ms. Eun Hee Han and Dr. Robert Sah for their contributions. This work was supported by National Institute of Health, National Science Foundation, and a predoctoral fellowship from the Whitaker Foundation for the dissertation author.
3.7 References


CHAPTER 4

SHORT-TERM RETENTION OF CHONDROCYTES IN STRATIFIED TISSUE-ENGINEERED CARTILAGINOUS CONSTRUCTS IMPLANTED IN VIVO IN MINI-PIGS

4.1 Abstract

The efficacy of cell-laden implants for cartilage defects likely depends on retention of implanted cells and interaction between implanted and host cells. The objectives of this study were to (1) characterize stratified cartilaginous constructs seeded with chondrocyte subpopulations labeled with PKH26 (*) and (2) determine the degree to which these stratified cartilaginous constructs maintain their architecture in vivo after implantation in mini-pigs for 1 week. Alginate-recovered cells were seeded to form S*/M and S*/M* constructs. Full-thickness defects (4 mm diameter) were created in the patellofemoral groove of adult Yucatan mini-pigs and filled with portions of constructs or left empty. Constructs were characterized biochemically, histologically, and biomechanically, and stratification visualized and quantified, before and after. After 1 week, animals were sacrificed and implants retrieved. GAG and COL content of constructs remained similar to that at implant while DNA content increased. Histological analyses revealed features of an early repair response with defects filled with tissues containing little matrix and abundant cells. Some implanted (PKH26-
labeled) cells persisted in the defects although constructs did not maintain a stratified organization. Of the labeled cells, 114±40% and 32±8% in S*/M and S*/M* constructs, respectively, were recovered. Distribution of labeled cells indicated interactions between implanted and host cells. Longer-term *in vivo* studies will be useful in determining whether implanted cells are sufficient to have a positive effect in repair.
4.2 Introduction

A number of tissue engineering studies have applied cells, scaffolds or materials, and growth factors, in combination or alone, to repair focal cartilage defects. Cells can be injected into a defect alone [5] or, for example, under a periosteal flap [9], within fibrin as a delivery vehicle [24], or within photopolymerizable hydrogel as a vehicle [18]. In addition, pre-formed, cell-laden tissues have been generated in vitro by seeding chondrocytes or chondroprogenitor cells on various types of scaffolds including those made of degradable synthetic materials [15, 17]. Homogeneous [22] and stratified cartilaginous tissues [31, 33] have been formed without a scaffold or as a cartilaginous layer as part of a composite scaffold [6, 54]. Purified or recombinant growth factors have been applied to cartilage defects in vivo alone [53], as part of a hydrogel designed to deliver the soluble signals [10, 36, 37], or encapsulated and then secreted by a biomaterial (fibrin glue or encapsulated liposome) designed to induce infiltration and retention of chondrogenic mesenchymal cells from the synovial membrane of the host [23, 26, 28]. Irrespective of the tissue engineering procedure, it is generally presumed that implanted cells, or host cells, of the appropriate phenotype, should be retained in sufficient number and in an organized fashion in the defect in order to fill the defect with newly synthesized extracellular matrix and to promote integration with the surrounding host tissues. However, cell retention can be transient [5, 43] presenting additional challenges.

The intrinsic repair response after subchondral bone penetration illustrates a number of cellular and matrix remodeling processes relevant to cell-based repair
therapies. Initiation of the repair process via creation of the defect causes formation of a blood clot followed by migration, adhesion and alignment of mesenchymal cells along a fibrinous arcade, and subsequent proliferation. Although by 12 weeks most of the tissue present appears to be well-organized, early signs of degeneration are observed with surface fibrillation and hypocellularity noted in the tissue filling the defect. Eventually, the repair tissue degenerates further with more extensive superficial fibrillation and vertical fissures observed [51]. These morphological features of the repair tissue are coincident with abnormal matrix content and inferior biomechanical properties. In comparison to the biochemical content of normal cartilage, the repair tissue contains less proteoglycan and significantly more type I collagen [21]. Analyses of the structural and functional abnormalities of repair cartilage suggest that a focus on cell fate processes may be required in order to design successful repair strategies.

Several in vivo studies have attempted to investigate cell fate processes underlying spontaneous self-repair and repair facilitated by injection of chondrocytes or implantation cell-laden tissues. The importance of investigating such processes has been highlighted by observations of cellular infiltration of partial-thickness defects treated enzymatically or with mitogenic growth factors [28], and of full-thickness defects treated with chondrocyte-seeded scaffolds [4, 43] or subchondral bone drilling [51]. In order to determine the number of donor cells recovered after implantation in vivo, chondrocytes expressing the sex-determining region Y gene, SRY [43], and chondrocytes transduced to express lacZ [4] have been used. With both methods, the spatial organization of implanted cells in the defect is unknown and the division
history of the implanted cells is difficult to assess. Implications of infiltration of implanted tissues for cartilage repair and tissue engineering strategies remain to be elucidated. However, it appears that a stable, long-lasting tracking method, applicable to localizing individual cells, would be useful in further investigating cell fate processes in vivo.

A stable family of lipophilic fluorescent dyes, developed previously for cell tracking, has begun to be used for tissue engineering. PKH dyes [25] are stably incorporated into the cell membrane, allowing for proliferation assessment [1, 2, 7, 12, 58], and appear to be non-toxic [1, 12, 20, 49]. PKH26-labeled chondrocytes were tracked through 7 cycles of cell division in vitro and cellular function of labeled cells was not adversely affected by labeling [12]. In addition, PKH26 has been applied to track cells implanted in vivo, including annulus fibrosus cells in rabbits [50] and chondrocytes in goats [16], and have been detected up to 3 months after implantation. Such applications indicate PKH26 could be useful for tracking chondrocytes in cell-laden tissues implanted in vivo.

Clarifying cell source and fates, by cell tracking, especially at early time-points after treatment of a cartilage defect, could lend insight into the processes underlying articular cartilage repair and aid in the development of tissue engineering strategies. Thus, the objectives of this study were to (1) generate and characterize cell-seeded stratified cartilaginous constructs with one or both chondrocyte subpopulations labeled with PKH26 and (2) determine the degree to which these stratified cartilaginous constructs maintain their architecture in a defect in vivo after implantation in the patellofemoral groove (PFG) for 1 week.
4.3 Materials and Methods

Approval for the study was obtained from the Institutional Animal Care and Use Committees (IACUC) of the University of California, San Diego and Rush University Medical Center.

Materials

Materials for cartilage explant, chondrocyte isolation, monolayer culture, PKH26 labeling, alginate bead culture, construct culture, biochemical procedures, biomechanical procedures, and immunohistochemical procedures were obtained as described previously [12, 14, 33]. Cal-EX II decalcification solution was from Fisher (St. Louis, MO). Fluka Biochemika Hematoxylin & Eosin (H&E), testicular hyaluronidase, and mouse monoclonal anti-collagen type I antibody were from Sigma (St. Louis, MO). Mouse monoclonal anti-collagen type II antibody cocktail was from Chemicon (Temecula, CA). Non-specific mouse monoclonal IgG antibody was from Pierce (Rockford, IL).

Experimental Design

To determine the degree to which generated stratified cartilaginous constructs maintained their architecture in a defect in the PFG after implantation in vivo, some chondrocytes were labeled with PKH26 (and subsequently designated as S* or M*) and used to generate S*/M and S*/M* constructs for implantation in vivo. Single (S*/M) and all-labeled (S*/M*) constructs were generated in order to determine the extent of infiltration by cells originating from the host while still detecting the degree of stratification maintained.
Primary Pre-culture, Labeling Chondrocytes with PKH26, and Formation of Constructs

Chondrocytes from the superficial (S) and middle (M) layers of immature (4-6 m.o.) porcine cartilage were isolated [12, 14, 33]. Articular cartilage slices from the S (<0.2 mm depth) and M (0.4-1.0 mm) layers were obtained from the PFG of sixteen immature (4-6 m.o.) Yucatan mini-pig knee joints (Sinclair Research Inc., Columbia, MO) and digested in medium (Dulbecco’s Modified Eagle Medium [DMEM], 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) with 0.2% pronase for 1 hour, and 0.02% collagenase-P for 16 hours.

S and M chondrocytes were expanded in monolayer culture as previously described [12]. Approximately 10 million released S or M chondrocytes were labeled with 20 μM PKH26 (and subsequently designated S* or M*) [12]. After labeling and washing, chondrocytes were cultured in 1.2% alginate beads [39] (4x10^6 cells/ml) in DMEM/F12 (20% fetal bovine serum [FBS], 25 μg/ml ascorbic acid with additives) and fed 1 ml medium per million cells per day.

After 14 days, cells with their associated matrix were released from alginate and resuspended in complete medium [39]. S* cells were seeded atop M or M* cells at an S:M proportion of 1:3 in Transwell® tissue culture inserts (12 mm diameter, 0.4 μm pore size polyester membrane) (Fig. 4.1) [33]. The final seeding density was 5 million cells/cm^2 membrane area. Cultures were maintained for an additional 4 weeks with medium changes every two to three days.
Figure 4.1. Schematic of methods used to generate stratified engineered constructs containing PKH26-labeled cells. Cells were isolated separately from superficial (S) and middle (M) zones of articular cartilage obtained from the PFG of 4-6 month old Yucatan mini-pigs and allowed to expand in low density monolayer culture. After release, some of the monolayer-expanded cells were labeled with PKH26 (indicated by *). Cells were cultured in alginate beads in order to allow formation of pericellular matrix and then released and seeded in tissue culture inserts. Two configurations were used: labeled S over non-labeled middle (S*/M) and labeled S over labeled M (S*/M*). These cultures were maintained for an additional 4 weeks to form cartilage constructs. Superficial (grey) and middle (white) zone chondrocytes are shaded differently to emphasize their location in culture.
Analyses of Tissues at Implant

Histological Analysis

Portions of *in vitro* generated constructs were histologically analyzed at the time. Samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned vertically 5 μm thick. In order to immunostain sections for type I and type II collagen, sections were de-paraffinized. Thereafter, sections were rinsed in hyaluronidase buffer (0.1M Na₂HPO₄, 0.15M NaCl, pH 5.3) and digested with testicular hyaluronidase (5,000 U/ml) for 30 minutes. Sections were probed for type I or type II collagen using monoclonal mouse anti-collagen I antibody or mouse anti-collagen II antibody cocktail, using the R.T.U. Vectastain Universal Elite ABC kit and Vector VIP peroxidase substrate kit. Other sections were probed with non-specific monoclonal mouse IgG antibody as a negative control. Sections of normal porcine articular cartilage and meniscus were both used as positive and negative controls. Results were documented by brightfield microscopy using a Nikon Eclipse TE 300 microscope (AG Heinze, Irvine, CA) equipped with SPOT RT camera (Diagnostic Instruments, Burlingame, CA).

Biochemical Characterization of Constructs

Portions of *in vitro* generated constructs were biochemically analyzed at the time of implant. Briefly, a 3 mm diameter disk was punched from each construct and solubilized with proteinase-K [33]. Portions of the digest were analyzed for DNA using PicoGreen® [40], sulfated glycosaminoglycan (GAG) by DMMB [19], and collagen (COL) by hydroxyproline [57]. DNA content was converted to cell number by using a conversion constant of 7.7 pg of DNA per cell [32]. Hydroxyproline
content was converted to collagen content by assuming a mass ratio of collagen to hydroxyproline equal to 7.14 [45, 57].

**Biomechanical Characterization of Constructs**

Portions of constructs were biomechanically analyzed after *in vitro* culture.

From some constructs, a specimen was isolated and tested in tension, as previously described [55]. A tapered specimen (1.8 mm wide and 4 mm long gage region) was isolated and subjected to tensile testing to determine tensile equilibrium, $E_{eq}$, and ramp, $E_{ramp}$, moduli, tensile strength, $\sigma_{ult}$, and failure strain, $\varepsilon_{ult}$ were determined.

From some constructs, a specimen was isolated and tested in confined compression as previously described [13, 56]. 9.6 mm diameter disks were isolated and subjected to equilibrium compression tests [13, 56] to determine the aggregate modulus, $H_{A0}$ for the whole tissue [34], the hydraulic permeability, $k_p$, and $M$, strain dependent permeability parameter [35].

**Visualization and Quantification of Stratification in Engineered Cartilage *in Vitro***

Portions of constructs at the time of implantation were fixed overnight in 2% paraformaldehyde, rinsed briefly, embedded in OCT compound, snap-frozen, and stored at -20°C until further analysis. 30 μm vertical cryosections were counterstained with 0.01 mg/ml Hoechst 33258 (H33258) to stain all cell nuclei. PKH26-labeled cells as well as nuclei of implanted and host cells were visualized by fluorescence microscopy (Fig. 4.2A).

Fluorescence micrographs of constructs at implantation were processed to determine the proportion of labeled and unlabeled cells as a function of depth from the
articulate surface (Matlab® 7.0 software, The MathWorks, Inc., Natick, MA) (Fig. 4.2A). Raw images were separated into blue (H33258) and red (PKH26) color channel images. All cell nuclei (blue) were isolated by filtering with a 5 x 5 Mexican hat filter [29, 30], color segmented using threshold values based on fluorescence intensity histograms, and separated by watershed algorithm [47]. PKH26-labeled regions were isolated using color segmentation and histogram analysis, and the resulting binary image was merged with the matching blue (H33258) binary image. Blue cell nuclei within red regions were considered PKH26-labeled cells, and other nuclei considered cells of host origin (Fig. 4.2B). For clarity, black background was replaced with white using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Images were divided into 10 equally sized bins through the full depth of the tissue, from the articular surface to subchondral bone, with labeled and unlabeled cell densities plotted as a function of depth from the articular surface (Fig. 4.2C). The number of cells per volume was determined by dividing the number of cells by the bin area and multiplying by the thickness of the section (30 µm).
Figure 4.2. Image processing of fluorescence micrographs of retrieved constructs containing PKH26-labeled cells and Hoecsht 33258 (H33258) stained nuclei of implanted and host cells. (A) The original image was read into a custom written Matlab® 7 subroutine. Blue and red color channels representing H33258 stained cell nuclei and PKH26-labeled cells respectively, were split and then individually filtered, normalized, and color segmented. The two segmented images were merged. (B) Blue nuclei (cells) within a red region were labeled cells, indicated by red circles encompassing blue nuclei and other nuclei were considered a cell of host origin. (C) The number of PKH26-labeled (red line) and total (blue line) cells per 10% bin were calculated and converted to cells per volume using the area of the tissue in each bin and the thickness of each section (30 μm).
The validity of the automated image processing method was assessed as previously described [30]. Manual methods of counting cell centroids present in higher magnification (20x) confocal fluorescence images were used as a gold standard. Cell nuclei positions from the automated and manual methods were overlaid in order to quantify inconsistencies in localization by the automated routine. 96.1% of identified cell nuclei were determined to be true positive (cell nuclei locations identified manually and automatically), 0.98% were false positive (cell nuclei locations identified automatically only), and 2.9% were false negative (cell nuclei locations identified manually only).

**Surgical Procedure and Analyses of Tissues at Retrieval**

Ten skeletally mature Yucatan mini-pigs 13-16 months old and weighing 80.3±1.7 kg (Sinclair Research Inc.) were implanted with cartilage constructs. Animals were given intramuscular injections of Telazol (4.4 mg/kg) and xylazine (2.2 mg/kg) followed by gas anesthesia (Isoflurane, 2-3% for induction, 1-2% for maintenance). The surgical site overlying the left knee joint was prepared for aseptic surgery with providone-iodine scrub solution and 70% isopropyl alcohol. A longitudinal skin incision was made proximal to the patella just medial of the tibial tuberculum. The investing layer was incised at the center of the patella and the joint was exposed by a medial parapatellar incision. The patella was dislocated medially to expose the PFG entirely. The joint surface was irrigated with saline periodically to avoid any drying of cartilage and other tissues.

The six experimental sites, separated by approximately 6 mm, were identified on the medial and lateral facets of the PFG. Five sites were used for 4 mm diameter
full-thickness defects created (Fig. 4.3) with a disposable biopsy punch, circular knife, and a surgical drill bit. In four of five defects, 4 mm diameter portions of the stratified constructs were implanted. The fifth defect was left empty and an additional sixth site within the joint was left intact as a “no defect” control (Fig. 4.3). The location of the no defect site, empty defect, as well as implanted constructs (S*/M and S*/M*), was randomly distributed in all animals. Constructs were affixed with two to four 7-0 Vicryl sutures. After implantation, the synovial joint capsule, medial retinaculum, subcutaneous tissue, and skin layers were closed. The right knee of each animal served as an unoperated contralateral control. After surgery, animals were allowed to recover and move freely without joint fixation. Postoperative pain was relieved with analgesics for a minimum of three days (buprenorphine, 0.01 mg/kg intramuscularly) and acetaminophen thereafter (0.3 g/kg pro re nata).

After surgery, no signs of infection or abnormal swelling were noticed upon visual inspection of the joint (daily post-surgery). One week after constructs were implanted, animals were sacrificed by injecting supersaturated phenobarbital solution (0.5 mg/kg intravenously). From each defect site, a 10 mm diameter osteochondral core, containing the implant, was retrieved (Fig. 4.3). From the contralateral knee, an unoperated control specimen from each of the six experimental sites was harvested.
Figure 4.3. Construct implantation and retrieval in Yucatan mini-pig. (A) 4 mm diameter portions of the stratified constructs were implanted into full thickness defects created in the PFG of skeletally mature Yucatan mini-pigs. One of five defects per operated knee was left empty and an additional site within the joint was left intact as a “no defect” control. (B) After 7 days, animals were sacrificed and 10 mm diameter osteochondral cores, containing the implant sites were assessed grossly en face (C), and sectioned vertically (D).
Histological Analysis

Portions of retrieved contralateral and implanted tissues were fixed, rinsed, decalcified in Cal-EX II for 6 weeks with daily changes, embedded in paraffin, and vertically sectioned to 5 μm. Sections were stained with non-specific mouse IgG, type I collagen, type II collagen antibodies as previously described as well as hematoxylin and eosin (H&E) and Safranin O/FastGreen [46].

Biochemical Characterization of Constructs

Retrieved constructs and host cartilage were characterized biochemically (DNA, GAG, COL assays) as described previously. Implant tissue was carefully dissected from one-quarter of the retrieved core and solubilized in proteinase-K. Overall amounts were normalized to volume, calculated as the product of the area of one-quarter of the implant region (4 mm diameter) and the thickness determined from fluorescence micrographs using Adobe Photoshop. Additionally, a 3 mm diameter disk was punched from host cartilage immediately adjacent to the implant site, digested, and analyzed biochemically.

Visualization and Quantification of Stratification in Engineered Cartilage in Vivo

Portions of retrieved cores containing implanted tissues were analyzed by fluorescence microscopy. Both the defect region and adjacent host cartilage were imaged. Normal cartilage retrieved from the contralateral knee was similarly analyzed. Images were processed as previously described (Fig. 4.2) to determine the number of labeled and unlabeled cells with depth from the articular surface. Fluorescence intensity associated with PKH26-labeled cells was controlled by a range of neutral density filters (reducing excitation light intensity by 1/2, 1/4, and 1/8) [12]. Based on
fluorescence intensity histogram analysis, three cycles of cell division were detectable above background levels, after implantation in vivo for 1 week, assuming the brightest cells were in generation 1 (data not shown). A portion of the retrieved core was used to confirm viability of the retrieved implant was greater than 95% viable (data not shown).

**Statistical Analysis**

Effects of sample type and time (at implant vs. at retrieval) on biochemical and biomechanical properties were assessed by ANOVA. Effects of sample type, time, and depth (bin) on cellularity and site and depth on thickness of control (non-operated) cartilage were assessed by repeated measures-ANOVA, $\alpha=0.05$ (Systat Software 10.2, Inc., Richmond, CA). Effect of sample type and time on thickness was assessed by ANOVA with post-hoc Tukey tests. Histological images were not statistically analyzed since results are described qualitatively. Data are expressed as mean ± SEM.

### 4.4 Results

**Analyses of Tissues at Implant**

**Histological Analysis**

Type II collagen, a major matrix constituent of normal articular cartilage, was detected throughout the engineered constructs. Sections probed for type I and type II collagens stained for type II collagen (Fig. 4.4IL) to a greater extent than type I (Fig. 4.4HK). Sections stained with non-specific mouse IgG antibody (negative control) were negative (Fig. 4.4GJ).
Figure 4.4. Histological assessment of (A–C) normal cartilage, (D–F) tissues filling empty defects, (G–I) S*/M and (J–L) S*/M* cartilaginous tissues at the time of implant and (M–O) S*/M and (P–R) S*/M* tissues after 1 week in vivo. Tissues were stained with (A, D, G, J, M, P) non-specific mouse IgG, (B, E, H, K, N, Q) type I collagen, or (C, F, I, L, O, R) type II collagen. Arrows indicate interface between defect and adjacent host cartilage. Bar=200 μm.
Biochemical Characterization of Constructs

After 4 weeks of *in vitro* culture, various indices of cell and matrix content were similar between S*/M and S*/M* constructs. DNA content of S*/M and S*/M* constructs were similar \((p=0.8)\) as were GAG content \((p=0.7)\), and COL content \((p=0.8)\) (Table 4.1).

Biomechanical Characterization of Constructs

Biomechanical properties of S*/M and S*/M* constructs were also similar after 4 weeks of culture. Tensile material properties of S*/M and S*/M* constructs were not significantly different \((p=0.22-0.86)\) (Table 4.2). The compressive modulus \((H_{\text{iso}})\) of S*/M and S*/M* constructs were similar \((p=0.9)\). The logarithmic fit values of hydraulic permeability \((k_p)\) also did not differ between construct types \((p=0.3)\) (Table 2). M, the strain dependent permeability function, did not vary between construct types \((p=0.6)\).
Table 4.1. Biochemical properties of S*/M and S*/M* cartilaginous tissues at the time of implantation.

<table>
<thead>
<tr>
<th></th>
<th>at implant</th>
<th></th>
<th>at retrieval</th>
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<tbody>
<tr>
<td></td>
<td>S*/M</td>
<td>S*/M*</td>
<td>normal cartilage empty</td>
</tr>
<tr>
<td>n:</td>
<td>5</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>DNA (mg/cm²)</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>n:</td>
<td>6</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>GAG (mg/cm²)</td>
<td>3.5 ± 0.7</td>
<td>2.1 ± 0.3</td>
<td>72.9 ± 6.1</td>
</tr>
<tr>
<td>n:</td>
<td>6</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>COL (mg/cm²)</td>
<td>7.8 ± 2.8</td>
<td>6.6 ± 4.0</td>
<td>198.9 ± 21.6</td>
</tr>
</tbody>
</table>
**Table 4.2.** Biomechanical properties of S*/M and S*/M* cartilaginous tissues at the time of implantation.

<table>
<thead>
<tr>
<th></th>
<th>at implant</th>
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<tbody>
<tr>
<td></td>
<td>S*/M</td>
<td>S*/M*</td>
<td></td>
</tr>
<tr>
<td>n:</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>$E_{eq}$ ($kPa$)</td>
<td>22 ± 5</td>
<td>15 ± 6</td>
<td></td>
</tr>
<tr>
<td>$E_{rang}$ ($kPa$)</td>
<td>69 ± 9</td>
<td>88 ± 32</td>
<td></td>
</tr>
<tr>
<td>$\sigma_{ult}$ ($kPa$)</td>
<td>59 ± 11</td>
<td>64 ± 20</td>
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<td>$\varepsilon_{ult}$ ($%$)</td>
<td>90 ± 14</td>
<td>66 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>$H_{A0}$ ($kPa$)</td>
<td>6 ± 2</td>
<td>7 ± 3</td>
<td></td>
</tr>
<tr>
<td>$\log_{10}K_p$ ($\varepsilon = 0%$)</td>
<td>-12.9 ± 0.4</td>
<td>-12.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>8 ± 1</td>
<td>7 ± 1</td>
<td></td>
</tr>
</tbody>
</table>
Visualization and Quantification of Stratification in Engineered Cartilage in Vitro

At the time of implant, stratification was qualitatively identified in S*/M constructs based on visualization of labeled and unlabeled regions. Additionally, PKH26-labeled cells were distributed uniformly throughout the depth of S*/M cartilage constructs and the number of labeled cells was not affected by depth from the articular surface (Fig. 4.5D and 4.7G, p=0.2). Quantitative analysis of the cells in S*/M cartilaginous constructs showed PKH26-labeled cells to be concentrated in the superficial 20% of the construct (p<0.05 to 0.001 compared to deeper regions), with 86% of cells in this region being identified as being PKH26-labeled (Fig. 4.5C and 4.7G). Total cellularity did not vary between construct types (p=0.73) nor was there an interactive effect of construct type and depth from the articular surface (p>0.9) (Fig. 4.7G). There was an independent effect of depth on cell density with significantly higher cell density in the upper 20% of the construct depth compared to deeper regions (p=0.001 to p<0.05) (Fig. 4.7G).
**Figure 4.5** Fluorescence micrographs of retrieved normal (control) cartilage (A) and tissue filling an empty defect (B) and constructs at the time of implantation (C, D) and at retrieval (E, F). (A) Normal cartilage tissue showed no PKH26 positive cells. (B) Non-implanted defects showed a thin layer of host cells, none being PKH26 positive. (C) A stratified S*/M implant showing PKH26-labeled cells localized at the surface of the construct. (D) A stratified S*/M* implant showing PKH26-labeled cells throughout the construct depth. Retrieved implants showed the presence of PKH26 labeled cells: (E) stratified S*/M implant, (F) stratified S*/M* implant.
Analyses of Tissues at Retrieval

Gross Assessment and Thickness

At retrieval, all defects, including those initially left empty, appeared to be filled with tissue and were flush with the surrounding adjacent host cartilage. S*/M and S*/M* constructs measured $1.3\pm0.1$ mm ($p=0.61$) at the time of implant and were slightly thicker than normal cartilage from the operated knee ($p<0.001$) (Fig. 4.7C). After 1 week in vivo, the S*/M repair tissue had a lower thickness, $0.86\pm0.1$ mm ($p<0.001$), while the S*/M* repair tissue thickness remained unchanged ($p=0.2$) (Fig. 4.7D). The thickness of both types of constructs at retrieval were similar to the thickness of control cartilage from both contralateral ($0.76\pm0.02$ mm) and operated knees ($0.78\pm0.03$ mm) as well as the thickness of tissue filling empty defects ($0.65\pm0.05$ mm) ($p=0.2-0.9$) (Fig. 4.7AB). In addition, there was no effect of site (lateral proximal, lateral middle, lateral distal, medial proximal, medial middle, medial distal) on thickness of cartilage retrieved from the contralateral knee ($p=0.9$) (Fig. 4.7A).

Histological Analysis

Histological and immunohistochemical differences were noted between normal cartilage and defects which had received implants or been left empty. Retrieved normal articular cartilage exhibited typical matrix staining (Fig. 4.4A-C, Fig. 4.6AB) by H&E, Safranin O/FG, and type I and II collagens. The entire depth of normal tissue stained intensely for proteoglycan (Fig. 4.6B) and type II collagen (Fig. 4.4C) with positive staining for type I collagen localized only in the uppermost superficial zone (Fig. 4.4B). In contrast, retrieved implants and tissues filling empty defects appeared
fibrous in nature (Fig. 4.6C-H) and did not exhibit Safranin O staining (Fig. 4.6DFH). The retrieved tissue was also negative for type I and II collagens (Fig. 4.4EF). Retrieved implants exhibited little type I collagen staining (Fig. 4.4NQ) while some type II collagen staining was noted in 5 out of 6 retrieved implants (3/3 S*/M and 2/3 S*/M*) and was distributed throughout the tissue depth (Fig. 4.4OR). Tissue sections stained with non-specific mouse IgG (negative control) antibody were negative for type I and II collagens (Fig. 4.4A, D, G, J, M, P).
Figure 4.6 Histological assessment of (A-B) normal cartilage, (C-D) tissues filling empty defects, (E-F) S*/M and (G-H) S*/M* cartilaginous tissues after 1 week in vivo. Tissues were stained with (A, C, E, G) hematoxylin & eosin (H&E) or (B, D, F, H) Safranin O/FastGreen (FG). Arrows indicate interface between defect and adjacent host cartilage. Bar=200 μm.
Figure 4.7 Thickness (A-D) and cellularity (E-H) of samples at implant and retrieval. (A) Thicknesses of control cartilage from the contralateral non-operated (non op’d) knee. Thicknesses of samples from the proximal (P), middle (M), and distal (D) sites from both lateral (LAT) and medial (MED) were obtained. (B) Thicknesses of control cartilage retrieved from the operated knee and tissue filling empty defects. (C) Thicknesses of S*/M and S*/M* constructs at time of implant. (D) Thicknesses of repair tissue from S*/M and S*/M* constructs at retrieval. (E) Cellularity with depth from the articular surface of control cartilage samples retrieved from the non-operated contralateral knee according to site. (F) Cellularity with depth from the articular surface of control cartilage from the operated (●) knee and cellularity of tissue filling empty defects (◊). (G) Total (□, Δ) and PKH26-labeled (■, ▲) cellularity of S*/M (□, ■) and S*/M* (Δ, ▲) at time of implant. (H) Total (□, Δ) and PKH26-labeled (■, ▲) cellularity of S*/M (□, ■) and S*/M* (Δ, ▲) at retrieval. Data are expressed as mean±SEM. P < 0.05 (♦)
Biochemical Characterization of Constructs

After 1 week in vivo, biochemical content of S*/M and S*/M* constructs did not differ significantly from each other or from tissues filling empty defects, but did differ significantly from biochemical content of normal cartilage and, for some biochemical indices, constructs at the time of implant (Table 4.1). DNA content of normal cartilage, S*/M and S*/M* constructs, and tissues filling empty defects at retrieval were similar (p=0.4). Constructs at retrieval were significantly higher in DNA content (S*/M 0.6±0.2 mg/cm³, S*/M* 0.7±0.2 mg/cm³) than constructs at the time of implant (S*/M 0.3±0.09 mg/cm³, S*/M* 0.1±0.04 mg/cm³; p<0.01). GAG content of constructs and tissues filling empty defects at retrieval were not different from each other (S*/M 2.7±0.6 mg/cm³, S*/M* 3.5±1.1 mg/cm³, empty 5.0±0.9 mg/cm³; p=1.0) or from constructs at the time of implant (S*/M 3.5±0.8 mg/cm³, S*/M* 2.1±0.2 mg/cm³, p=0.8) but were significantly lower in GAG content than normal cartilage (72.9±6.0 mg/cm³, p=0.001 – p<0.01). Finally, COL content of constructs and tissues filling empty defects were similar (p=0.4) to each other as well as constructs at the time of implant (p=0.4). COL content of retrieved tissues (S*/M 6.7±0.3 mg/cm³, S*/M* 6.7±1.1 mg/cm³, empty 11.8±1.3 mg/cm³) was significantly lower than normal cartilage (198.9±22.0 mg/cm³, p<0.05 – p=0.05).

Visualization and Quantification of Stratification in Engineered Cartilage in Vivo

Retrieved control cartilage from both contralateral (unoperated) and operated knees (“no defect” site) were similar in cellularity and showed no PKH26 positive cells (Fig. 4.7A). Normal cartilage retrieved from the “no defect” site in the operated knee exhibited decreased cellularity with depth from the articular surface (p<0.001
upper 10% vs. lower bins) and was not significantly different grossly, or in variation of cellularity with depth from the articular surface, from normal cartilage retrieved from the unoperated contralateral knee (Fig. 4.7E). In addition, there was not a significant effect of site on the cellularity with depth of normal cartilage retrieved from contralateral knees (p=0.12; Fig. 4.7E). There was also not a significant interactive effect of aspect (lateral vs. medial) and type of defect treatment on cellularity with depth of adjacent host cartilage (p=0.52).

Tissues filling empty (non-implanted) defects were similar in total cellularity to S*/M and S*/M* constructs (p=0.2). Tissues filling empty defects contained no PKH26 positive cells and exhibited uniform host cellularity compared to the depth-varying cellularity observed of control cartilage (p<0.001 for all depths) (Fig. 4.7F). Retrieved implants showed the presence of PKH26 labeled cells in both S*/M* and S*/M constructs (Fig. 4.6EF and 4.7H) although stratification was not maintained in vivo. After 1 week in vivo, distribution of PKH26-labeled cells in S*/M and S*/M* constructs were similar (p=0.72) as were the total number of cells per volume present in the two types of retrieved implants (p=0.15). The cellularity of PKH26-labeled cells was somewhat lower than the overall cellularity at the defect site indicating influx of many additional host cells (Fig. 4.7G, p<0.01 S*/M; p=0.001 S*/M*). PKH26-labeled cells were not observed in adjacent host cartilage.

Some PKH26-labeled implanted cells were retained and persisted in the defect region after implantation in vivo for 1 week (Fig. 4.8). At the time of implant, S*/M constructs contained 150±31% of the labeled cells initially seeded. The number of labeled cells in S*/M retrieved tissues was similar, 126±38% (p>0.9). In contrast,
S*/M* constructs at implant contained 93±17% of the labeled cells initially seeded and the number of labeled cells at retrieval after 1 week decreased to 32±8% of the number initially seeded (p<0.05).
Figure 4.8 Percentage of PKH26-labeled cells detected at implantation (□, Imp) after 1 week in vivo (■, Ret) relative to the number of cells initially seeded. Dashed lines represent the number of PKH26-labeled cells initially seeded at the time of construct formation. Data are mean±SEM (n=6-9). $P < 0.01$ (▲)
4.5 Discussion

The above results indicate that labeled chondrocyte subpopulations can persist for 1 week in cartilage defects in vivo after implantation. Cell-laden tissue-engineered stratified constructs, containing PKH26-labeled chondrocytes, were generated in vitro (Fig. 4.1), implanted in vivo in small defects created in the PFG of adult mini-pigs (Fig. 4.3), retrieved, and labeled implanted cells tracked (Figs. 4.5, 4.7, 4.8). At the time of implant, stratification was present in S*/M constructs, based on visualization of PKH26-associated fluorescence at the surface of constructs (Fig. 4C) and quantification of the number of labeled and unlabeled cells with depth from the surface (Fig. 4.2, 4.7G). At the time of implant, S*/M and S*/M* constructs were similar in thickness (Fig. 4.7C), biochemical content (Table 4.1), and biomechanical properties (Table 4.2). The repair response after 1 week appeared typical, with defects containing highly cellular and low matrix tissues (Fig. 4.6C-H). Retrieved constructs were similar in thickness to normal cartilage (Fig. 4.7AC) but histologically dissimilar (Figs. 4.4, 4.6). Biochemically, retrieved construct tissues contained more DNA than those at implant. GAG and COL content of retrieved tissues were similar to those at implant and lower than normal articular cartilage (Table 4.1). After implantation in vivo for 1 week, some labeled implanted cells were retained and persisted in vivo (Figs. 4.5, 4.7, 4.8).

Localization of PKH26-labeled chondrocyte subpopulations after implantation in vivo allowed for examination of cellular organization and distribution at an early time point (1 week) and provided insight into processes underlying initial repair responses. In the future, longer durations in vivo, implantation of multi-labeled and/or
different types of cartilage constructs, would be useful in further elucidating a mechanistic understanding of cartilage repair in vivo. Although some aspects of the zonal architecture of articular cartilage have been recapitulated by generating stratified cartilaginous tissues in vitro [31, 33], comparing the repair quality of cartilage defects after treatment with stratified cartilage constructs versus traditional cartilage constructs made up of mixed chondrocyte subpopulations, or no treatment, could indicate potential efficacy of cartilaginous tissues tailored to exhibit stratification like native cartilage. In this study, cartilaginous tissue was formed from culture-expanded chondrocytes, in contrast to the tissue formed previously from bovine cartilage with cells that were not expanded and from newborn animals [33], and resulted in tissues that were thin and possibly more susceptible to infiltration by host-derived cells. Stimulating cultures with BMP-7 has enhanced construct formation [38] without affecting maintenance of stratification in vitro [11]. Such robust constructs could be implanted in vivo to further assess the roles of S and M cells in the repair of articular cartilage defects.

An advantage to creating multiple defects in the PFG of each animal was the ability to assess and develop the cell tracking methodology in an efficient manner. Based on visualization of PKH26-labeled cells, there did not appear to be any direct interactions between neighboring defects since no implanted, i.e. tagged, cells were not observed at sites of normal tissue or empty defects. Additionally, normal cartilage retrieved from unoperated contralateral knees was similar both grossly and in cellularity with depth from the articular surface to cartilage retrieved from “no defect” sites in operated knees. In order to avoid possible effects of defect location in the joint,
samples were randomly distributed in each knee. Any exposure to soluble signals (from the synovial fluid) or joint loading on the defects would likely have been received similarly among all samples especially since there are few site differences in the (bovine) PFG in terms of biomechanical properties [55, 56]. Finally, several other studies have studied repair of multiple defect sites, both in the PFG or femoral condyle in a variety of animal models including mini-pigs [27], dogs [8], goats [41], horses [42], and rabbits [52]. It should be noted that the use of multiple defects in this study was intended for a mechanistic study, not for an outcome study meant to closely mimic the clinical situation.

Applying a quantitative method to in vivo tracking studies, such as the method developed in this study, allowed for direct assessment of cellularity and cellular organization and distribution. Cellularity trends of control cartilage, as assessed by the imaging processing method here, generally agreed with those found previously both by 3D imaging techniques [30] and biochemical quantification by DNA assay [56]. In this study, image analysis returned relatively low numbers of false positive and false negative cells. Higher resolution image analysis, coupled with a long-lasting, stable tracker dye, such as PKH26, could likely be used in development of models for predicting cellular fates such as migration, proliferation, and cell death.

Although implanted tissues were somewhat fragile in nature, localized regions of type II collagen staining in retrieved implants (Fig. 4.4OR), and lack of type II collagen staining in defects initially left empty (Fig. 4.4F), indicated persistence of implanted cells in vivo possibly contributing to defect repair. Defects appeared to contain tissues which were highly cellular and likely a mix of both implanted and
infiltrating host cells, supporting previously reported studies in rabbits [5] and goats [43]. In addition, fibrous synovium-like tissue appeared to reside at the surface of defect regions treated with S*/M or S*/M* constructs (Fig. 4.6EFGH) or no treatment (empty) (Fig. 4.6CD). In the present study, defect repair quality was not histologically graded due to the early time point, when differences among defect treatments (S*/M, S*/M*, empty) were likely to not be evident. Further, defects treated with construct implants compared to defects initially left empty, did not qualitatively appear to differ in terms of H&E staining (Figure 4.6EG) and contained tissues that were devoid of proteoglycan-associated Safranin O staining (Fig. 4.6FH).

In this study, interactions between implanted and host cells appear to be present. A lower percentage of labeled cells were retained in defects treated with S*/M* (32%) compared to S*/M constructs (126%). This could imply loss of M* labeled cells to the subchondral bone region or active proliferation of M* cells in vivo leading to diminishment of fluorescence and therefore, lack of visualization. Additionally, the low matrix content of the implanted construct tissues could have contributed to their susceptibility to infiltration by host cells since increased extracellular matrix production has been shown to enhance donor cell retention [3]. Infiltration of cartilage defects by host-derived cells, and in some cases, concomitant loss of implanted cells, has been noted in defects treated by a variety of methods. Partial-thickness defects treated enzymatically or with mitogenic growth factors [28], as well as full-thickness defects treated by chondrocyte-seeded scaffolds [4, 43], or subchondral bone drilling [51] have been observed to be infiltrated by host cells. The source of such host cell influx is believed to be primarily from the synovial lining.
(synoviocytes and subsynovial mesenchymal cells) [28] and bone marrow sinuses [44, 51]. Creation of the cartilage defect caused cell death in the immediate periphery of the defect (noted in the present study as well as previously published studies [48, 51]), resulting in a region which remained either acellular or filled with chondrocyte clones. Injections of $^3$H-thymidine and $^3$H-cytidine in rabbits indicated lack of participation of chondrocytes in the adjacent host cartilage to repair of the defect [51]. Along with host cell influx, implanted cell loss is likely to be at least partially affected by mechanical forces associated with the implantation procedure and also joint motion (impact loading, compression, shear) [4, 44].

Implications of infiltration of implanted tissues for cartilage repair and tissue engineering strategies, as well as the mechanism(s) underlying infiltration, remain to be determined. Infiltration of immature implants observed in the present study indicates potent contributions from both implant and host cells to the population of the defect and suggests the possibility of modulating the extent of infiltration in vivo by varying properties of implanted constructs. Further clarification of the roles of implanted and host cells could have implications for tissue engineering strategies aimed at cartilage repair.

### 4.6 Acknowledgments

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CHAPTER 5

STRATIFICATION OF CARTILAGINOUS
TISSUE IMPLANTS WITH LABELED CHONDROCYTE
SUBPOPULATIONS: EFFECTS ON EARLY IN VIVO
REPAIR OF ARTICULAR DEFECTS

5.1 Abstract

Objective: To treat cartilage defects with stratified or mixed constructs or no implant and compare the (1) depth-associated distribution and density of implant-derived cells, (2) quality of repair after 4 weeks in vivo, and relationships between (1) and (2).

Design: Superficial (S) and middle (M) zone porcine chondrocytes were-expanded, some labeled with PKH26 (S*), and then cultured in alginate. After release, M cells were labeled with CFSE (M^). Cells were seeded sequentially (S*/M^) or mixed (S*+M^). Full-thickness defects (4 mm diameter) were created in the patellofemoral groove of adult Yucatan mini-pigs and filled with portions of constructs or left empty. Constructs at the time of implant (t=0) were characterized biochemically, histologically, biomechanically, and fluorescently. Tissues at retrieval (t=4 wk) were similarly analyzed.

Results: At t=4 wk, S*/M^ constructs had a surface region containing S* cells and few M^ cells. Unlabeled cells were present in both S*/M and S*+M^ constructs at t=4
Tissues at $t=0$ and 4 wk exhibited positive staining for type II collagen and GAG. Surface roughness of $S^*+M^*$ constructs was higher than $S^*/M^*$ constructs. Surface roughness of tissues filling empty defects was higher than normal cartilage.

**Conclusions:** Interactions between host and implant at both the articular and subchondral surfaces may markedly affect cell retention and infiltration. $S^*/M^*$ constructs maintained certain features of stratification after 4 weeks in vivo which were distinct from $S^*+M^*$ constructs. The phenotypic differences between chondrocyte subpopulations and construct types may affect cartilage repair.
5.2 Introduction

A number of strategies for treating cartilage defects rely on delivery of implanted cells. Cells can be injected into a defect alone [6] or, for example, under a periosteal flap [8], as in autologous chondrocyte transplantation, or within fibrin [26] or photopolymerizable hydrogel as a delivery vehicle [19]. In addition, pre-formed, cell-laden tissues have been generated \textit{in vitro} by seeding chondrocytes or chondroprogenitor cells on various types of scaffolds including those made of degradable synthetic materials [14, 18]. Purified or recombinant growth factors have been applied to cartilage defects \textit{in vivo} alone [54], as part of a hydrogel designed to deliver the soluble signals [9], or encapsulated and then secreted by a biomaterial (fibrin glue or encapsulated liposome) designed to induce infiltration and retention of chondrogenic mesenchymal cells from the synovial membrane of the host [25, 29, 30].

Efficacy of cell-laden implants for repair of articular cartilage defects likely depends on the retention of implanted cells and interactions between implanted and host cells. Implanted cells, or host cells, of the appropriate phenotype, should be retained in sufficient number and in an organized fashion in the defect in order to fill the defect with newly synthesized extracellular matrix and to promote integration with the surrounding host tissues.

Experimental studies have shown that implanted cells may not be retained in the defect site. Using various tagging methods and cell delivery methods, the number of recovered, tagged cells, relative to the number implanted, can be low as early as one day after the implant [6, 7, 22, 42]. Recovery appears to decrease with increased
time in vivo [17] but can be enhanced by pre-incubation of cells in carrier scaffolds[4], or incubation to pre-form tissue constructs [11]. It is likely that cells at the defect are derived both from efflux of implanted cells as well as influx of host cells, and the proportion of implant-derived cells relative to other cells may modulate the efficacy of defect repair.

Since articular cartilage exhibits zonal differences, stratified cartilaginous constructs tailored to recapitulate such an architecture have been introduced [33, 36]. The result is a tissue with zonal properties recapitulating normal articular cartilage, especially with chondrocytes in the superficial zone secreting the lubricant, proteoglycan 4 (PRG4) [50] and increasing GAG and collagen with depth from the articular surface [35]. Organization and phenotype of cells in cartilage constructs may partially modulate the quality of cartilage repair. Thus, it would be useful to localize and determine the relative retention of implanted cells of different phenotypes in vivo.

Tracking methods have been implemented using fluorescent dyes that allow localization of individual cells. PKH dyes [28] are stably incorporated into the cell membrane, allowing for proliferation assessment [12], and appear to be non-toxic [2, 12, 21, 49]. PKH26 has previously been applied to track chondrocytes implanted in vivo, by autologous chondrocyte transplantation [17], and implant of stratified cartilaginous tissues containing PKH26-labeled subpopulations [11]. Application of most fluorescent cytoplasmic dyes (CellTracker™ Green CMFDA, CellTracker™ Orange CMTMR, BCECF-AM), for tracking cells and determining cellular organization is limited to short-term studies lasting only a few days [24] due to dye dilution with cellular division. A longer-lasting cytoplasmic dye is carboxyfluorescein
diacetate, succinimidyl ester (CFDA, SE)[43] which passively diffuses into cells and is highly fluorescent and well-retained once its acetate groups are cleaved by intracellular esterases [43]. CFSE can also be applied to track generations of cells by dye dilution due to division and appears to be retained within the cell [23] without adverse effects on cell function [55]. Such applications indicate PKH26 and CFSE to be useful for tracking chondrocytes in cell-laden tissues implanted in vivo.

The hypothesis of this study was that the efficacy of cell-laden implants depends on (1) the persistence of implanted cells as well as (2) the depth-associated organization of superficial and middle cells in implanted constructs. To test this, the objectives of this study were to treat cartilage defects with constructs either stratified (S*/M^) like normal cartilage, or composed of a mixed population of cells (S*+M^), or no implant, and then compare the depth-associated distribution and density of implant-derived cells, the quality of repair after 4 weeks in vivo, and the relationships between implant-derived cells and repair quality.

5.3 Materials and Methods

Approval for the study was obtained from the Institutional Animal Care and Use Committees (IACUC) of the University of California, San Diego and Rush University Medical Center.

Materials

Materials for cartilage explant, chondrocyte isolation, monolayer culture, PKH26-labeling, alginate bead culture, construct culture, biochemical procedures, biomechanical procedures, and immunohistochemical procedures were obtained as
described previously [12, 36]. Cal-EX II decalcification solution, Tissue-Tek OCT, Centricon® filter (100kDa cutoff), mouse monoclonal anti-collagen type I antibody, Fluka Biochemika Hematoxylin & Eosin (H&E), and testicular hyaluronidase were from Sigma (St. Louis, MO). CFDA-SE (CFSE) labeling kit was from Invitrogen/Molecular Probes (Carlsbad, CA). Mouse monoclonal anti-collagen type II antibody cocktail was from Chemicon (Temecula, CA). Non-specific mouse monoclonal IgG antibody was from Pierce (Rockford, IL). Ready Gel Tris-HCl 4-15% gels were from Bio-Rad (Hercules, CA). Polyvinylidene fluoride (PVDF) membrane and DEAE Sepharose™ gel were from Amersham Biosciences (Piscataway, NJ)

Experimental Design

To determine the effects of $S^*/M^*$ and $S^*+M^*$ implants or no implant on repair quality after implantation in vivo for 4 weeks, chondrocytes were labeled with PKH26 (and subsequently designated $S^*$) or CFSE (designated $M^*$) and used to generate $S^*/M^*$ and $S^*+M^*$ constructs for implantation in vivo.

Primary Pre-culture, Labeling Chondrocytes with PKH26 or CFSE, and Formation of Constructs

Chondrocytes from the superficial (S) and middle (M) layers of immature (4–6 m.o.) porcine cartilage were isolated [12, 36]. Articular cartilage slices from the S ($<0.2$ mm depth) and M (0.4–1.0 mm) layers were obtained from the PFG of sixteen immature (4–6 m.o.) Yucatan mini-pig knee joints (Sinclair Research Inc., Columbia, MO) and digested in medium (Dulbecco’s Modified Eagle Medium [DMEM], 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100
U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) with 0.2% pronase for 1 hour, and 0.02% collagenase-P for 16 hours.

S and M chondrocytes were expanded in monolayer culture as previously described [12]. Approximately 10 million released S chondrocytes were labeled with 20 μM PKH26 (and subsequently designated S*) [12]. M cells were left unlabeled. After labeling and washing, chondrocytes were cultured in 1.2% alginate beads [40] (4x10^6 cells/ml) in DMEM/F12 (20% FBS, 25 μg/ml ascorbic acid, 50 ng/ml bone morphogenetic protein-7 (BMP-7) [10, 39] with additives) and fed 1 ml medium per million cells per day.

After 14 days, cells with their associated matrix were released from alginate and resuspended in complete medium [40]. M cells were labeled with CFSE (and subsequently designated M^). Approximately 40 million M cells were washed once in medium, pelleted, and resuspended in 1 ml of phosphate buffered saline (PBS) containing the CFSE probe (final concentration 20 μM [23]) according to manufacturer’s directions. Chondrocytes were incubated with the labeling solution for 5 min at 25°C. The staining reaction was stopped by adding an equal volume of FBS and incubating for 1 min. Cells were washed and pelleted twice thereafter, each time with 5 ml complete medium.

S* and M^ chondrocytes in suspension were seeded sequentially or mixed in 12 mm Transwell® tissue culture inserts (0.4 μm pore size polyester membrane) in order to form stratified (S*/M^) [36] and mixed (S*+M^) constructs, respectively (Fig. 5.1). Cells were seeded at an S:M proportion of 1:3 in Transwell® tissue culture inserts (12 mm diameter, 0.4 μm pore size polyester membrane) (Fig. 5.1) [36]. The
final seeding density was 5 million cells/cm² membrane area. Cultures were maintained for an additional 4 weeks with medium changes every two days.
Figure 5.1. Schematic of methods used to generate stratified and mixed tissue engineered constructs containing PKH26-labeled and CFSE-labeled cells. Cells were isolated separately from superficial (S, grey) and middle (M, white) zones of articular cartilage obtained from the patellofemoral groove of 4-6 month old Yucatan mini-pigs and allowed to expand in low density monolayer culture. After release, S cells were labeled with PKH26 (indicated by *). Cells were cultured in alginate beads in order to allow formation of pericellular matrix and then released. M cells were labeled with CFSE (indicated by ^). Cells were seeded sequentially or in combination in tissue culture inserts to form stratified (S*/M^) and mixed (S*+M^) cartilage constructs. These cultures were maintained for an additional 4 weeks to form cartilage constructs. Superficial (grey) and middle (white) zone chondrocytes are shaded differently to emphasize their location in culture.
Analyses of Tissues at Implant (t=0)

Histological Analysis

Portions of in vitro generated constructs were histologically analyzed at the time of implant (t=0). Samples were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned vertically 5 μm thick. In order to immunostain sections for type I and type II collagen, sections were de-paraffinized. Thereafter, sections were rinsed in hyaluronidase buffer (0.1M Na₂HPO₄, 0.15M NaCl, pH 5.3) and digested with testicular hyaluronidase (5,000 U/ml) for 30 minutes at 37°C. Sections were probed with monoclonal mouse anti-collagen I antibody or mouse anti-collagen II antibody cocktail, using the R.T.U. Vectastain Universal Elite ABC kit and Vector VIP peroxidase substrate kit. Other sections were probed with non-specific monoclonal mouse IgG antibody as a negative control. Sections of normal porcine articular cartilage and meniscus were both used as positive and negative controls. Serial sections were also stained with Safranin O/FastGreen (FG) and H&E. Results were documented by brightfield microscopy using a Nikon Eclipse TE 300 microscope (AG Heinze, Irvine, CA) equipped with SPOT RT camera (Diagnostic Instruments, Burlingame, CA).

Brightfield images of constructs immunohistochemically stained for type I or II collagens at t=0 were processed to determine the proportion of tissue area positive for either collagen type as a function of depth from the articular surface (Matlab® 7.0 software, The MathWorks, Inc., Natick, MA). RGB images were converted to CMYK. The surface and bottom of the construct, and non-tissue regions (gaps or holes resulting from sectioning) were traced in Adobe Photoshop 7.0 (Adobe Systems, San
Jose, CA). The magenta channel was isolated and color segmented using threshold values based on color intensity histograms in order to indicate regions positive for type I or II collagen.

In order to determine the volumetric density of cells (#/cm³) as a function of relative depth from the articular surface, the articular surface and bottom surface tracings were digitized, and a best fit line to each of these landmarks was determined. The height of the articular cartilage was determined as the average distance between these lines. Images were divided into 10 equally sized bins through the full depth of the tissue, top to bottom surface. The percentage positive for type I or II collagen was determined by dividing the area positive by the total tissue area, determined by subtracting non-tissue areas from the bin area. Resulting values were adjusted for false positive staining in sections stained with non-specific mouse IgG antibody.

Biochemical Characterization of Constructs

Portions of in vitro generated constructs were biochemically analyzed at the time of implant (t=0). Briefly, a 3 mm disk was punched from each construct and solubilized with proteinase-K [36]. Portions of the digest were analyzed for DNA using PicoGreen® [41], sulfated glycosaminoglycan (GAG) by DMMB [20], and collagen (COL) by hydroxyproline [58]. DNA content was converted to cell number by using a conversion constant of 7.7 pg of DNA per cell [34]. Hydroxyproline content was converted to collagen content by assuming a mass ratio of collagen to hydroxyproline equal to 7.14 [45, 58].

Biomechanical Characterization of Constructs

Portions of constructs were biomechanically analyzed at t=0.
From some constructs, a specimen was isolated and tested in tension, as previously described [56]. A tapered specimen (1.8 mm wide and 4 mm long gage region) was isolated and subjected to tensile testing to determine tensile equilibrium, $E_{eq}$, and ramp, $E_{ramp}$, moduli, tensile strength, $\sigma_{ult}$, and failure strain, $\varepsilon_{ult}$ were determined.

From some constructs, a specimen was isolated and tested in confined compression as previously described [13, 57]. 9.6 mm diameter disks were isolated and subjected to equilibrium compression tests [13, 57] to determine the aggregate modulus, $H_{A0}$ for the whole tissue [37], the hydraulic permeability, $k_p$, and M, strain dependent permeability parameter [38].

**Visualization and Quantification of Stratification in Engineered Cartilage in Vitro**

Portions of constructs at $t=0$ were fixed overnight in 2% paraformaldehyde in phosphate buffered saline (PBS), rinsed briefly, embedded in OCT compound, snap-frozen by immersion in liquid nitrogen cooled isopentane, and stored at -20°C until further analysis. Samples were vertically sectioned to 200 μm on a freezing stage (Physitemp Instruments Inc., Clifton, NJ) mounted on a sledge microtome (Microm Instruments, Waldorf, Germany). Sections were counterstained with 0.01 mg/ml Hoechst 33258 in PBS to stain all cell nuclei, and imaged in 3-D by confocal laser scanning microscopy (BioRad MRC 1024) with a 10X, 0.45 NA objective. S* and M^ cells, as well as nuclei of implanted and host cells were visualized.

Confocal fluorescence images of constructs at $t=0$ were processed to determine the proportion of labeled and unlabeled S and M cells as a function of depth from the articular surface. Images were imported into Matlab® 7.0 software (The MathWorks,
Inc., Natick, MA). The surface and bottom of the construct, and non-tissue regions (gaps or holes resulting from sectioning) were traced in Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). Raw images were separated into red (PKH26), green (CFSE), and blue (H33258) color channel images. All cell nuclei (blue) were isolated by filtering with a 5 x 5 Mexican hat filter [31], color segmented using threshold values based on fluorescence intensity histograms, and separated by watershed algorithm [47]. PKH26 (red) and CFSE (green)-labeled regions were isolated using color segmentation based on histogram analysis and merged with the matching H33258 (blue) processed image. For qualitative viewing, black background in images was replaced with white using Adobe Photoshop. Blue cell nuclei within either a red (PKH26) or green (CFSE) region were considered a labeled (implanted) cells and nuclei that were not within a red or green region were considered unlabeled.

In order to determine the volumetric density of cells (#/cm³) as a function of relative depth from the articular surface, the articular surface and bottom surface tracings were digitized, and a best fit line to each of these landmarks was determined. The height of the articular cartilage was determined as the average distance between these lines. Images were divided into 10 equally sized bins through the full depth of the tissue, top to bottom surface. The number of cells per volume was determined by dividing the number of cells by the tissue volume, determined by subtracting non-tissue areas from the bin area and multiplying by the estimated depth of field of the tissue section (60 μm). Labeled and unlabeled cell densities were plotted as a function of depth from the articular surface.
The validity of the automated image processing method was assessed by comparing automated methods of localizing labeled and unlabeled cells to manual methods (used as a gold standard). Cell nuclei positions from the automated and manual methods were overlaid in order to quantify inconsistencies in localization by the automated routine [32]. 96.1% of identified cell nuclei were determined to be true positive (cell nuclei locations identified manually and automatically), 0.98% were false positive (cell nuclei locations identified automatically only), and 2.9% were false negative (cell nuclei locations identified manually only).

**Surgical Procedure and Implantation of Cartilage Constructs In Vivo**

Eight skeletally mature Yucatan mini-pigs 14-18 months old and weighing 61.6±2.6 kg were used. Animals were allowed to acclimatize for 1 week prior to surgery. The surgical procedure was conducted as previously described [11]. Briefly, animals were given intramuscular injections of Telazol (4.4 mg/kg) and xylazone (2.2 mg/kg) followed by gas anesthesia (Isoflurane, 2-3% for induction, 1-2% for maintenance). The surgical site overlying the left knee joint was prepared for aseptic surgery with providone-iodine scrub solution and 70% isopropyl alcohol. A longitudinal skin incision was made proximal to the patella just medial of the tibial tuberculum. The investing layer was incised at the center of the patella and the joint was exposed by a medial parapatellar incision. The patella was dislocated medially to expose the PFG entirely. The joint surface was irrigated with saline periodically to avoid any drying of cartilage and other tissues.

The three experimental sites, separated by approximately 6 mm, were identified on the lateral facet of the patellofemoral groove (Fig. 5.2). Three sites were
used for 4 mm diameter full-thickness defects created (Fig. 5.2) with a disposable biopsy punch, circular knife, and a surgical drill bit. Four mm diameter portions of the stratified and mixed constructs were press fit into two of the three full thickness defects. The third defect was left empty (Fig. 5.2). The location of the empty defect, as well as implanted constructs (S*/M^ and S*+M^), was randomly distributed in all animals. Constructs were affixed with two to three 7-0 Vicryl sutures. After implantation, the synovial joint capsule, medial retinaculum, subcutaneous tissue, and skin layers were closed. The right knee of each animal served as an unoperated contralateral control. After surgery, animals were allowed to recover and move freely without joint fixation. Postoperative pain was relieved with analgesics for a minimum of three days (buprenorphine, 0.01 mg/kg intramuscularly) and acetaminophen thereafter (0.3 g/kg pro re nata).

Four weeks after constructs were implanted, animals were sacrificed by injecting supersaturated phenobarbital solution (0.5 mg/kg) and intact joints were collected immediately (t=4 wk). From each defect site, a 10 mm diameter osteochondral core, containing the implant, surrounding host cartilage, and underlying bone was retrieved (Fig. 5.2). From the contralateral knee, an unoperated control specimen from each of the three experimental sites was harvested.
Figure 5.2. Construct implantation and retrieval in skeletally mature Yucatan mini-pigs. (A) 4 mm diameter portions of the stratified constructs were implanted into full thickness defects created in the patellofemoral groove. One of three defects per operated knee was left empty. (B) After 4 weeks, animals were sacrificed and 10 mm diameter osteochondral cores, containing the implant sites were assessed grossly en face (C), and sectioned vertically (D).
**Analyses of Tissues at Retrieval (t=4 weeks)**

**Gross Inspection**

At the time of retrieval, tissues were assessed visually and sectioned vertically, to be distributed among various analyses groups.

**Histological Analysis**

Portions of retrieved contralateral and implanted tissues were fixed, rinsed, decalcified in Cal-EX II for 6 weeks with daily changes, embedded in paraffin, and vertically sectioned to 5 μm. Sections were stained with non-specific mouse IgG, type I collagen, type II collagen antibodies as previously described as well as H&E and Safranin O/FG [46]. Images of sections stained for non-specific mouse IgG, type I collagen, and type II collagen were quantified for collagen staining as previously described.

Sections stained with Safranin O/FastGreen were evaluated with a newly developed semi-quantitative histological grading system [51, 59] and applied with image processing software (ImageJ, Bethesda, MD). The sections were evaluated blindly by three independent observers (KC, JH, NTH). The scale consisted of 8 distinct categories which assessed the quality of repair tissue in the defect, the surface roughness of the repair tissue, the degree of bonding at the interface, and the bone (Table 5.1). For the repair tissue, the thickness of tissue filling the defect was quantified and the degree of matrix staining, as determined by extent of Safranin O dye retention, and presence of cartilaginous features, i.e., whether the tissue appeared to be hyaline or fibrocartilage or a combination, were assessed. The root mean square surface roughness was calculated as the deviation of the actual surface profile (traced
by independent observers) from an idealized surface. In the present study, the idealized surface consisted of a third order polynomial curve fit to the actual surface profile [59]. The percentage of bonding at the interface between the repair tissue and adjacent host cartilage of the tissue was also quantified by drawing line tracings using Adobe Photoshop and measuring the lengths of these lines with ImageJ software. The remaining two categories were required for assessment of the subchondral bone region. These were the amount of residual cartilage present in the deeper regions of the cartilage defect and the percentage reformation of the tidemark. Areas in the bone tissue which histologically stained as cartilage (red, Safranin O stain) were selected, quantified with ImageJ software, and normalized to the width of the image. The percentage reformation of the tidemark was quantified with the same procedure as that applied for determining the percentage of bonding at the interface.

Biochemical Characterization of Constructs

Constructs were characterized biochemically (DNA, GAG, COL assays) at the time of retrieval \(t=4\text{ wk}\) as described previously. Implant tissue was carefully dissected from one-quarter of the retrieved core (containing implant tissue) and solubilized in proteinase-K overnight. Overall amounts were normalized to volume, calculated as the product of the area of one-quarter of the implant region (4 mm diameter) and the thickness determined from fluorescence micrographs using Adobe Photoshop. Additionally, a 3 mm diameter disk was punched from host cartilage immediately adjacent to the implant site, digested, and analyzed biochemically.
Table 5.1. Semi-quantitative grading scale for assessment of articular cartilage repair

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>thickness [μm]</td>
<td>area of filled defect normalized to width of defect region</td>
<td>0</td>
</tr>
<tr>
<td>Safranin O staining</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal</td>
<td>red (Safranin O)</td>
<td>4</td>
</tr>
<tr>
<td>slightly reduced</td>
<td>predominantly red with few green (FastGreen) regions</td>
<td>3</td>
</tr>
<tr>
<td>moderately reduced</td>
<td>even mix of red and green regions</td>
<td>2</td>
</tr>
<tr>
<td>substantially reduced</td>
<td>predominantly green with few red (Safranin O) regions</td>
<td>1</td>
</tr>
<tr>
<td>none</td>
<td>green (FastGreen)</td>
<td>0</td>
</tr>
<tr>
<td>Cartilaginous features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hyaline cartilage</td>
<td>repair tissue appears the same as normal cartilage, including cellular</td>
<td>4</td>
</tr>
<tr>
<td>mostly hyaline cartilage</td>
<td>morphology and organization</td>
<td></td>
</tr>
<tr>
<td>mostly fibrocartilage</td>
<td>&gt;50% of tissue is fibrocartilage</td>
<td>3</td>
</tr>
<tr>
<td>mostly non-cartilage</td>
<td>&gt;50% of tissue is fibrocartilage</td>
<td>2</td>
</tr>
<tr>
<td>non-cartilage only</td>
<td>defect is empty or contains only inflammatory material</td>
<td>0</td>
</tr>
<tr>
<td>roughness [μm]</td>
<td>Root mean square (RMS) roughness determined based on differences between</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the repair profile and the idealized surface profile</td>
<td></td>
</tr>
<tr>
<td>bonded [**]</td>
<td>Summed areas of bonded segments normalized to area of total interface segment</td>
<td>0</td>
</tr>
<tr>
<td>residual cartilage [μm²/μm]</td>
<td>Summed areas of residual cartilage normalized to the width of the defect</td>
<td>0</td>
</tr>
<tr>
<td>tidemark reformation [**]</td>
<td>Summed areas of reformed segments normalized to area of normal tidemark</td>
<td>0</td>
</tr>
</tbody>
</table>
Visualization and Quantification of Stratification in Engineered Cartilage in Vivo

Portions of retrieved cores containing implanted tissues or tissues filling empty defects were fixed overnight and processed as previously described. Samples were vertically sectioned to 100 μm on a freezing stage and counterstained with 0.01 mg/ml Hoechst 33258 in PBS to stain all cell nuclei, and imaged in 3-D by confocal laser scanning microscopy. Both the defect region and adjacent host cartilage were imaged. Normal cartilage retrieved from the contralateral knee was similarly analyzed. Images were processed as previously described in order to determine the number of labeled and unlabeled cells in the defect region with depth from the articular surface to the cartilage-bone interface. A portion of the retrieved core was used to confirm viability of the retrieved implant was greater than 95% viable (data not shown).

Statistical Analysis

Effects of sample type and bin were assessed by ANOVA with Tukey post-hoc tests. Repeated measures-ANOVA was used to assess the effect of depth with planned comparisons. Histopathological scores were analyzed with the Kruskal-Wallis test. Data are expressed as mean±SEM.

5.4 Results

Analyses of Tissues at Implant (t=0)

Gross Assessment and Thickness

At t=0, S*/M^ and S*+M^ constructs were easy to handle (Figure 5.3A1, A2) and similar in thickness (1.4±0.1 mm, p=1.0; Figure 5.3B).

Histological Analysis
Tissues at the time of implant appeared to stain for matrix constituents, such as glycosaminoglycans and type II collagen. S*/M^ and S*+M^ constructs were thick, as depicted by H&E staining (Fig. 5.4A-i, -ii) and histologically exhibited Safranin O staining for GAG content (Fig. 5.4B-i, -ii). In addition, constructs were stained positively for type II collagen (Fig. 5.4E-i, -ii) and were negative for type I collagen (Fig. 5.4D-i, -ii). Sections stained with non-specific mouse IgG antibody (negative control) were negative for type I and II collagens (Fig. 5.4C-i, -ii).

Quantification of the distribution of type II collagen indicated differences between construct types with depth from the surface (Fig. 5.4E-i,ii; Fig. 5.5A). At $t=0$, S*/M^ constructs contained significantly less type II collagen at the uppermost surface of the construct compared to S*+M^ constructs (Fig. 5.4E-i,ii; Fig. 5.5A) (p<0.001). Constructs were similarly negative for type I collagen at $t=0$ (Fig. 5.4D-i,ii; Fig. 5.5A) (p=0.6)

Biochemical Characterization of Constructs

After 4 weeks of in vitro culture, biochemical content of S*/M^ and S*+M^ constructs did not differ significantly. DNA content of S*/M^ and S*+M^ constructs were similar (S*/M^ 0.11±0.01 mg/cm^3, S*+M^ 0.13±0.02 mg/cm^3; p=0.3; Fig. 5.3C) as were GAG content (S*/M^ 16.4±2.1 mg/cm^3, S*+M^ 11.8±2.6 mg/cm^3; p=0.2; Fig. 5.3D), and COL content (S*/M^ 3.7±1.3 mg/cm^3, S*+M^ 1.6±0.5 mg/cm^3; p=0.2) (Fig. 5.3E).

Biomechanical Characterization of Constructs

Biomechanical properties of S*/M^ and S*+M^ constructs were similar after 4 weeks of culture. Tensile material properties (equilibrium modulus: 99.8±45.4 kPa,
dynamic modulus: 239.7±84.0 kPa, tensile strength: 71.2±25.3 kPa, and failure strain: 50.2±11.0 %) of S*/M and S*/M* constructs were not significantly different (p=0.2-0.6) (Table 5.2).

The compressive modulus ($H_{A0}$) of S*/M and S*/M* constructs measured 1.5±0.2 kPa (p=0.3). The hydraulic permeability ($k_p$) measured at 0% strain also did not differ between construct types (-10.6±0.3, p=0.6) (Table 5.2). $M$, the strain dependent permeability function, did not vary between construct types (14.9±1.2, p=0.4).
Figure 5.3 Effects of defect treatment ((A1) S*/M^, (A2) S*+M^, empty) and time (t=0 (white) or t=4 wk (black)) in comparison to retrieved normal cartilage (striped) and tissues filling empty defects (grey) on (B) thickness and biochemical content: (C) DNA, (D) GAG, (E) COL. Data are mean ± SEM, n = 4-24. P < 0.001 (▲), P < 0.01 (●), P < 0.05 (♦)
**Figure 5.4** Histological assessment of (A-i:E-i) $S^*/M^*$ and (A-ii:E-ii) $S^*+M^*$ cartilaginous tissues at the time of implant, (A-iii:E-iii) normal cartilage, (A-iv:E-iv) tissues filling empty defects, and (A-v:E-v) $S^*/M^*$ and (A-vi:E-vi) $S^*+M^*$ tissues at retrieval. Tissues were stained with (A-i:vi) hematoxylin & eosin (H&E), (B-i:vi) Safranin O/FastGreen (Saf O/FG), or antibodies for (C-i:vi) non-specific mouse IgG, (D-i:vi) type I collagen, or (E-i:vi) type II collagen. Arrows indicate interface between defect and adjacent host cartilage.
Figure 5.5 Effects of defect treatment ($S^*/M^+$ (■, □); $S^*+M^+$ (▲, △); empty (◇, ◤)), and time ($t=0$ (A) or $t=4$ wk (B, C)), compared to retrieved normal cartilage (●, ○) and tissues filling empty defects, on type I (□, △, ◤, ○) and II (■, ▲, ◇, ●) collagen distribution in comparison to. Data are mean ± SEM, $n = 2-5$. 
Table 5.2 Biomechanical properties of S*/M^ and S*/+M^ constructs at the time of implantation.

<table>
<thead>
<tr>
<th></th>
<th>$t=0$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S*/M^</td>
<td>$S*/+M^$</td>
</tr>
<tr>
<td>n:</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>$E_{eq}$ ( (kPa))</td>
<td>$49 \pm 20$</td>
<td>$130 \pm 91$</td>
</tr>
<tr>
<td>$E_{ramp}$ ( (kPa))</td>
<td>$214 \pm 110$</td>
<td>$255 \pm 151$</td>
</tr>
<tr>
<td>$\sigma_{ult}$ ( (kPa))</td>
<td>$53 \pm 23$</td>
<td>$82 \pm 49$</td>
</tr>
<tr>
<td>$\varepsilon_{ult}$ ( (%))</td>
<td>$70 \pm 28$</td>
<td>$38 \pm 4$</td>
</tr>
<tr>
<td>n:</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>$H_{A0}$ ( (kPa))</td>
<td>$2 \pm 0$</td>
<td>$1 \pm 0$</td>
</tr>
<tr>
<td>$\log_{10} k_p$ ( (\varepsilon = 0%))</td>
<td>$-10 \pm 1$</td>
<td>$-11 \pm 0$</td>
</tr>
<tr>
<td>M</td>
<td>$16 \pm 2$</td>
<td>$14 \pm 1$</td>
</tr>
</tbody>
</table>
Visualization and Quantification of Stratification in Engineered Cartilage in Vitro

S* and M^ cell density each varied significantly with depth from the articular surface (p<0.001), depending on the implant type (interaction p<0.001), but not with t (S*, p=0.5; M^, p=0.3) (Fig. 5.8AG). At t=0, in the superficial region, PKH26-label was evident in 42±4% of all cells, and in deeper regions, CFSE-label was apparent in 9±1% of all cells (Fig. 5.8AI). In contrast, S*+M^ constructs at t=0 showed no significant depth-variation in labeled cells (p=0.3) (Fig. 5.8BGI). Overall cell densities were 5.8±0.8 10^6 cells/cm^3 for S* cells, and 2.6±0.3 10^6 cells/cm^3 for M^ cells.

Analyses of Tissues at Retrieval (t=4 wk)

Gross Assessment and Thickness

After surgery, animals were in good health and behaved normally. No signs of infection or abnormal swelling were noticed upon visual inspection of the joint (daily post-surgery).

At t=0, S*/M^ and S*+M^ constructs were significantly thicker than normal cartilage obtained from the contralateral knee (Fig. 5.3B, p<0.01). At retrieval, all defects, including those initially left empty, appeared to be filled with tissue and were flush with the surrounding adjacent host cartilage. At t=4 wk, the thickness of retrieved constructs was significantly less than at t=0 (p<0.01 vs. S*/M^, p<0.05 vs. S*+M^) and were similar in thickness to adjacent cartilage and tissues filling empty defects (0.8±0.04 mm, p>0.9; Fig. 5.3B).

Biochemical Characterization of Constructs

Biochemical content varied between tissues at the time of implant compared to retrieval and generally differed from that of normal porcine articular cartilage. DNA
content of retrieved constructs was 4.5-fold higher than normal cartilage (p<0.05) and 4.7-fold higher than constructs at \( t=0 \) (p<0.001) (Fig. 5.3C). GAG content of retrieved constructs was 2.5-fold lower than at \( t=0 \) (p<0.01), but equivalent to tissues retrieved from empty defects (p>0.9) (Fig. 5.3D). GAG content of retrieved tissues was 9-fold lower than normal cartilage (p<0.01) (Fig. 5.3D). COL content of retrieved constructs was 19-fold higher than at \( t=0 \) (p<0.001-0.01), and equivalent in content to normal cartilage and tissues filling empty defects (p=0.7) (Fig. 5.3E).

**Histological Analysis**

Based on Safranin O staining and tissue type (Table 5.3), the \( t=0 \) implants resembled native cartilage more than the repair tissue at \( t=4 \) wk. While native cartilage (Fig. 5.4B-iii) was distinguishable from \( S^*+M^* \) constructs (Fig. 5.4B-vi, p<0.05) and tissues filling empty defects (Fig. 5.4B-iv, p<0.05) in terms of matrix staining, \( S^*/M^* \) (Fig. 5.4B-v) treatment was not statistically different (p=0.2). Native cartilage and repair tissues at \( t=4 \) wk showed significant differences in the percentage of bonded interface (Table 5.3, p<0.05) although the percentage of bonded interface was similar between \( S^*/M^* \) (75±11%) and \( S^*+M^* \) (54±28%) (p=0.6). Additionally, the absence of a reformed tidemark was noted in all retrieved tissues (\( S^*/M^*, S^*+M^*, \) empty) compared to native cartilage (Fig. 5.4B-iii:vi, p<0.001).

Variability of GAG and type I and II collagen staining was noted among retrieved tissues of the same type (Fig. 5.6). At \( t=4 \) wk, some \( S^*/M^* \) tissues stained for GAG (as assessed by Safranin O/FG) throughout the depth of the defect (Fig. 5.6B-i) while others exhibited GAG staining in localized regions (Fig. 5.6B-ii:iii),
which seemed to coincide with regions of positive staining for type II collagen (Fig. 5.6E-ii:iii).

Quantification of the distribution of type II collagen indicated differences between defect treatments and native cartilage (Fig. 5.4E-iii-vi; Fig. 5.5BC). At \( t=4 \) wk, distribution of type II collagen in \( S^*/M^\) (Fig. 5.4E-v; Fig. 5.5C) and \( S^*+M^\) (Fig. 5.4E-vi; Fig. 5.5C) constructs appeared to be similar (\( p=0.2 \)) but were significantly different from native cartilage (\( p<0.001 \) – \( p<0.05 \)) (Fig. 5.4E-iii; Fig. 5.5B). Distribution of type II collagen was also similar between tissues filling empty defects (Fig. 5.4E-iv; Fig. 5.5B) and \( S^*+M^\) (Fig. 5.4E-vi; Fig. 5.5C) constructs (\( p>0.6 \)) however, there appeared to be some differences between tissues filling empty defects (Fig. 5.4E-iv; Figure 5.5B) and \( S^*/M^\) (Fig. 5.4E-v; Fig. 5.5C) constructs with depth from the articular surface (\( p=0.06 \) for bins 6 and 9, all others \( p>0.1 \)). Distribution of type I collagen was similar among retrieved tissues and native cartilage (\( p=0.8 \)) with depth from the articular surface (\( p=0.2 \)).

Defect treatment (\( S^*/M^\), \( S^*+M^\), empty) had an effect on the surface roughness of the repair tissue after 4 weeks \textit{in vivo}. Surface roughness of \( S^*+M^\) constructs (Fig. 5.7B) at the time of implant was 1.9-fold higher than \( S^*/M^\) constructs (Fig. 5.7A) (\( p<0.01 \)). After 4 weeks \textit{in vivo}, tissues filling empty defects (Fig. 5.7D) had a rough surface, 6-fold higher than that of normal cartilage (Fig. 5.7C) (\( p<0.05 \)). Similarly, retrieved \( S^*+M^\) (Fig. 5.7E) tissues had higher surface roughness (2.7 fold higher) compared to retrieved \( S^*/M^\) (Fig. 5.7F) tissues (\( p<0.01 \)).
Figure 5.6 Histological assessment of S*/M* cartilaginous tissues at the time of retrieval. Staining for (A-i:iii) H&E, (B-i:iii) Safranin O/FG, (D-i:iii) Col I, and (E-i:iii) Col II are somewhat variable among three different S*/M* retrieved. Arrows indicate interface between defect and adjacent host cartilage.
**Table 5.3** Histopathological characteristics of samples.

<table>
<thead>
<tr>
<th>exp group</th>
<th>type</th>
<th>n</th>
<th>repair tissue in defect</th>
<th>surface</th>
<th>interface</th>
<th>bone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>thickness [μm]</td>
<td>Safranin O staining</td>
<td>Cartilaginous features</td>
<td>roughness [μm]</td>
</tr>
<tr>
<td>0</td>
<td>S'/M⁺</td>
<td>3</td>
<td>260 ± 15</td>
<td>3.0 ± 0.0</td>
<td>2.7 ± 0.0</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>0</td>
<td>S⁺M⁺</td>
<td>4</td>
<td>310 ± 59</td>
<td>2.9 ± 0.1</td>
<td>2.7 ± 0.0</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>normal cart.</td>
<td>7</td>
<td>270 ± 8</td>
<td>3.8 ± 0.2</td>
<td>4.0 ± 0.0</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>empty</td>
<td>3</td>
<td>109 ± 31</td>
<td>1.0 ± 0.6</td>
<td>1.6 ± 0.2</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>4</td>
<td>S'/M⁺</td>
<td>5</td>
<td>202 ± 19</td>
<td>2.1 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>S⁺M⁺</td>
<td>2</td>
<td>314 ± 108</td>
<td>0.8 ± 0.2</td>
<td>1.3 ± 0.0</td>
<td>11 ± 5</td>
</tr>
</tbody>
</table>
Figure 5.7 Effects of construct type and defect treatment on surface roughness. Actual (black, solid line) and idealized (orange, dashed line) surface tracings from Safranin O/FastGreen images of (A) $S^*/M^*$ and (B) $S^*+M^*$ constructs at the time of implant (A, B) and retrieval (C, D) as well as (E) normal contralateral tissues and (F) tissues filling empty defects at retrieval. (G) Surface roughness was defined as the degree of deviation from an idealized, smooth surface. Root mean square surface roughness measurements were made for tissues at $t=0$ and 4 wk. Data are mean ± SEM, n = 2-5. $P < 0.01$ (●)
Visualization and Quantification of Stratification in Engineered Cartilage in Vitro

At retrieval, construct stratification continued to show significant effects on cell organization. At \( t=4 \text{ wk} \), \( S^*/M^\) constructs maintained some stratification with the surface region containing \( S^* \) cells and relatively few \( M^\) cells, much fewer than the \( M^\) cells in the surface region of \( S^*+M^\) constructs (\( p<0.05-0.09 \), Fig. 5.8EFJ). In contrast, the distribution of \( S^* \) cells in \( S^*/M^\) and \( S^*+M^\) was similar (\( p=0.9 \)) at \( t=4 \text{ wk} \). Overall cell densities in \( S^*/M^\) constructs was \( 11.6\pm1.4 \times 10^6 \ \text{cells/cm}^3 \) for \( S^* \) cells, \( 5.7\pm0.8 \times 10^6 \ \text{cells/cm}^3 \) for \( M^\) cells, and \( 32.3\pm3.5 \times 10^6 \ \text{cells/cm}^3 \) for unlabeled cells. Overall cell densities in \( S^*+M^\) constructs was \( 11.9\pm3.2 \times 10^6 \ \text{cells/cm}^3 \) for \( S^* \) cells, \( 7.4\pm1.3 \times 10^6 \ \text{cells/cm}^3 \) for \( M^\) cells, and \( 25.9\pm1.5 \times 10^6 \ \text{cells/cm}^3 \) for unlabeled cells (\( p=0.3-0.9 \)). At \( t=4 \text{ wk} \), Densities for \( S^* \), \( M^\), and unlabeled cells were similar (\( p=0.3-0.9 \)) between construct types. Tissues filling empty defects contained \( 36.2\pm8.8 \times 10^6 \ \text{cells/cm}^3 \). Native cartilage and tissues filling empty defects did not contain any cells with PKH26 or CFSE (Fig. 5.8CD).

As indicated by cellularity profiles (Fig. 5.8G-J), some implanted PKH26 and CFSE-labeled cells were retained in the defect region after implantation in vivo for 4 weeks. The number of \( S^* \) cells retained in \( S^*/M^\) and \( S^*+M^\) retrieved tissues was similar (\( p>0.9 \)) (Fig. 5.9). There appeared to be a trend to an effect of time (\( p=0.1 \)) in vivo on the retention of PKH26-labeled cells in the defect site. Relative to the number of PKH26-labeled cells present at \( t=0 \), 68% were recovered in \( S^*/M^\) retrieved tissues and 48% in \( S^*+M^\) retrieved tissues at \( t=4 \text{ wk} \) (Fig. 5.9A). There was not a significant interaction of construct type and time (\( p=0.6 \)). The number of \( M^\) cells retained in \( S^*/M^\) and \( S^*+M^\) retrieved tissues was also similar (\( p=0.3 \)). Relative to the number
of CFSE-labeled cells present at $t=0$, 47% were recovered in S*/M^ retrieved tissues and 20% in S*+M^ retrieved tissues, significantly lower than the number of CFSE-labeled cells present at $t=0$ ($p<0.01$) (Fig. 5.9B). There also appeared to be a trend towards a significant interaction between construct type and time in vivo ($p=0.1$).
Figure 5.8 (A-F) Processed confocal images of samples of indicated type and t to localize PKH26-labeled (red), CFSE-labeled (green), and other (blue) cells. Bar=100 µm. (G,I) Cellularity of PKH26-labeled cells (red square, red triangle) and CFSE-labeled cells (green square, green triangle) in S*/M^ (red square, green square) and S*+M^ (red triangle, green triangle) constructs, and cellularity relative to all cells (blue square, blue triangle), as a function of depth from the tissue surface. (H) Cellularity of normal cartilage (●) from the contralateral (unoperated) knee, normal host cartilage from the operated knee (○), and tissues filling empty defects (♦). Data are mean±SEM, n=3-6.
Figure 5.9 Percentage of (A) PKH26-labeled and (B) CFSE-labeled cells detected at implantation (□) and after 4 weeks in vivo (■) for S*/M^ and S*+M^ constructs. Data are mean±SEM (n=3-6).
5.5 Discussion

In this *in vivo* study, the effects of treating articular cartilage defects with stratified or mixed tissue constructs or no implant (empty defect) on the depth-associated distribution and density of implant-derived cells and quality of defect repair after 4 weeks *in vivo* was investigated. Cell-laden tissues containing PKH26-labeled (*S*) and CFSE-labeled (*M*) chondrocytes, were generated *in vitro* (Fig. 5.1), implanted *in vivo* in small defects created in the PFG of adult mini-pigs (Fig. 5.2), retrieved, and labeled implanted cells localized (Figs 5.8 and 5.9). At the time of implant, stratification was present in *S*/*M* constructs, based on visualization of PKH26-associated fluorescence at the surface of constructs and CFSE-associated fluorescence in deeper regions of the constructs (Fig. 5.8A). At the time of implant, *S*/*M* and *S*/*M* constructs were similar in thickness (Fig. 5.3B), biochemical content (Fig. 5.3CDE), and biomechanical properties (Table 5.2). Histologically, *S*/*M* constructs (Fig. 5.4BE-*) appeared to contain less GAG and type II collagen near the surface compared to *S*+*M* constructs (Fig. 5.4BE-ii). Additionally, *S*/*M* constructs had a smoother surface compared to *S*+*M* constructs, both at the time of implant and retrieval (Fig. 5.7ABEFG), After 4 weeks *in vivo*, DNA and COL content of retrieved tissues had increased while GAG content decreased. Histological differences in the repair response of defects treated with cartilaginous tissues versus left empty were evident, with retrieved implants containing localized positive staining for type II collagen (Fig. 5.4A-v:E-v, Fig. 5.5C), while tissue filling empty defects did not.
Localization of PKH26-labeled and CFSE-labeled chondrocyte subpopulations after implantation \textit{in vivo} allowed for examination of cellular organization and distribution at a relatively early time point (4 weeks) and provided insight into processes underlying initial repair responses. Quantitative cellularity distribution data of the number of labeled and unlabeled cells with depth from the surface (Fig. 5.8GI) further supported qualitative observations. In the future, longer durations \textit{in vivo}, larger sample size, and implantation of constructs in a more mechanically challenging location, e.g., femoral condyle versus patellofemoral groove, would be useful in further elucidating a mechanistic understanding of cartilage repair \textit{in vivo}. In this study, cultures were stimulated with BMP-7 in order to enhance construct formation [39] without affecting maintenance of stratification \textit{in vitro} [10]. While constructs were more robust than previously generated stratified porcine constructs, tissues remained somewhat soft. Mechanical properties could be enhanced prior to implantation with application of dynamic stimulation \textit{in vitro} although the minimum level of mechanical integrity required for tissue engineered implants is unknown. In this study, mechanical characterization of tissue at the time of retrieval was not conducted in order to preserve tissue organization and structure. However, it would certainly be useful to explore this in the future. Finally, although constructs were not 100% labeled at the time of implant, likely due to proliferation-associated dye dilution \textit{in vitro}, a significant proportion of labeled cells were recovered and localized relative to the number implanted indicating persistence and trackability of implanted cells.

Histological assessment revealed features of the early repair response after creation of a defect and implantation of cartilaginous tissues \textit{in vivo}. Tissues at the
time of implant were thick (Fig. 5.3B) and stained for proteoglycan and type II collagen in localized regions throughout the construct depth (Fig. 5.4BE-i; 5.4BE-ii, 5.5A). Retrieved constructs contained localized regions of type II collagen staining (Fig. 5.4E-v, 5.4E-vi, Fig. 5.5C), and little type I staining, while tissues filling initially defects initially left empty lacked type II collagen staining (Fig. 5.4E-iv), indicating contribution and persistence of implanted cells of chondrogenic phenotype to defect repair. Semi-quantitative grading of defect quality indicated some differences in some histological indices among defect treatments (S*/M^, S*+M^, no treatment (empty defect)). In particular, matrix staining scores indicated greater similarity between normal cartilage and retrieved S*/M^ tissues compared to S*+M^ and tissues filling empty defects. Interface bonded similar among groups Defects appeared to contain tissues which were highly cellular and likely a mix of both implanted and infiltrating host cells, supporting previously reported studies in rabbits [6], goats [42], and mini-pigs [11]. In addition, fibrous tissue appeared to reside at the surface of defect regions initially left empty (Fig. 5.4AB-iv).

Implant-host cell interactions appear to be present in vivo which will likely have an effect on implanted cell retention, and may have a role in the modulation of cartilage repair. Loss of implanted cells and invasion of cartilage defects by host-derived cells has been noted in full-thickness defects treated by chondrocyte-seeded scaffolds [5, 42], as well as defects treated with subchondral bone drilling [52]. The source of such host cell influx is believed to be primarily from the synovial lining (synoviocytes and subsynovial mesenchymal cells) [30] and bone marrow sinuses [44, 52]. Injections of ^3H-thymidine and ^3H-cytidine in rabbits indicated lack of
participation of chondrocytes in the adjacent host cartilage to repair of the defect [52].

Creation of the cartilage defect caused cell death in the immediate periphery of the defect (noted in the present study as well as previously published studies [48, 52]), resulting in a region which remained either acellular or filled with chondrocyte clones. Along with host cell influx, implanted cell loss is likely to be at least partially affected by mechanical forces associated with the implantation procedure and also joint motion (impact loading, compression, shear) [5, 44]. In this study, a lower percentage of CFSE-labeled cells were recovered after implantation in vivo relative to the number of CFSE-labeled cells present initially. This could imply loss of M^ labeled cells to the subchondral bone region or active proliferation of M^ cells in vivo leading to diminishment of fluorescence and therefore, lack of visualization. Implications of infiltration of implanted tissues for cartilage repair and tissue engineering strategies, as well as the mechanism(s) underlying infiltration, remain to be determined.

S*/M^ maintained certain features of stratification after 4 wk in vivo that were distinct from S*+M^ constructs. The greater density of M^ cells at the surface of S*+M^ constructs compared to S*/M^ constructs, as well as the increased surface roughness of S*+M^ constructs compared to S*/M^ constructs, suggests implications of cell phenotype in cartilage repair. This finding is in part supported by other studies which have indicated distinct phenotypic differences between S and M cells, including differences in morphology [1], cell diameter [27], biosynthetic activity [3, 53], matrix content [15, 27, 53, 60], proliferation [3, 27], and viscoelastic mechanical properties [16]. In addition, chondrocytes originating from the superficial zone of cartilage are known to secrete the lubricant molecule PRG4 (also termed superficial zone protein
(SZP) [50]). Superficial chondrocytes at the surface of S*/M^ constructs could have a protective effect at the surface of the defect region and prevent surface roughening. Further analysis of PRG4 secretion and immunolocalization in vivo, as well as other markers of cell phenotype, are necessary in order to better understand the role of S and M chondrocytes in repair of articular cartilage defects.

After implantation in vivo for 4 week, some labeled implanted cells were retained with some aspects of chondrocyte stratification maintained after 4 weeks in vivo suggesting a relationship between localized retention of implanted (labeled) cells of particular phenotype and repair quality. In addition, different construct types were applied to cartilage defects and resulted in some differences in repair quality, providing further motivation for recapitulation of the zonal architecture of articular cartilage in tissue engineering of cartilage constructs.

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CHAPTER 6

CONCLUSIONS

6.1 Abstract

The findings of the dissertation are summarized and related to potential future in vitro and in vivo studies needed to characterize the efficacy of engineered tissues for cartilage repair. Other cartilage tissue engineering techniques are presented as well as some key criteria for successful repair of focal cartilage defects.
6.2 Summary of Findings

The objectives of this dissertation were to establish cytotracking methods for chondrocytes and to use these methods to assess the cellular mechanisms of articular cartilage repair \textit{in vivo} after implantation of cartilaginous tissue composed of two chondrocyte subpopulations. Constructs were either stratified, resembling normal cartilage, or composed of a mixed population of cells. The results of these studies provided insight into mechanisms underlying articular cartilage repair \textit{in vivo} (Fig. 1.6).

The effects of fluorescent tracker dyes on chondrocyte proliferation and function (matrix content, secretion of PRG4) were determined. Additionally, a generation model was used to analyze the proliferation history of labeled chondrocytes. High retention of PKH26 (Chapter 2) and CFSE (Chapter 3) fluorescence label by chondrocytes from superficial and middle zones as well as the lack of adverse effects of cell labeling on cell proliferation and synthesis of PRG4 (and for CFSE, matrix content) suggested that the dyes would be useful in determining the fate and function of implanted chondrocytes \textit{in vivo}, as well as monitoring proliferation \textit{in vitro}.

The interactions present between implanted (labeled) and host cells \textit{in vivo} were evident after 1 week \textit{in vivo} (Chapter 4). Stratified cartilaginous constructs seeded with one or both chondrocyte subpopulations labeled with PKH26 were implanted \textit{in vivo} in the patellofemoral groove of Yucatan mini-pigs. After 1 week, repair tissue was highly cellular and contained little GAG. Some implanted (PKH26-labeled) cells persisted in the defects although constructs did not maintain a stratified organization. From these studies, it appeared that a certain degree of maturity was needed in the construct prior to implantation \textit{in vivo}. Increased GAG content and
thickness of constructs cultured with bone morphogenetic protein – 7 (BMP-7) [15], along with improved handleability of constructs, prior to implantation showed promise for improvement of implanted cells and maintenance of stratified structure (Appendix A) [3].

Longer-term in vivo studies were needed to determine whether implanted cells were sufficient to have a positive effect on repair in vivo. In addition, in applying different defect treatments (stratified vs. mixed constructs, compared to no treatment [empty defect]) consisting of two chondrocyte subpopulations organized two ways, implementing a second tracker dye was important. Dual-labeled constructs were generated using PKH26 and CFSE-labeled chondrocytes in either stratified or mixed form (Chapter 5). A significant proportion of implant-derived chondrocytes were retained in the defect region after 4 weeks in vivo although infiltration of the cartilage defects by host cells occurred. After 4 weeks, stratified constructs maintained certain features of stratification which were distinct from mixed constructs. Additionally, differences in repair quality, specifically matrix staining and surface roughness, indicated a relationship between repair quality and the cell organization and cell phenotype of the implant.

6.3 Future Studies

Several studies are necessary to further understand the mechanisms underlying cartilage repair in vivo as well as establish the efficacy of cartilaginous tissues for cartilage repair. These include both in vitro and in vivo studies.

The work presented here established cytotracking methods using PKH26 (Chapter 2) and CFSE (Chapter 3). Both tracking dyes brightly labeled chondrocytes, and lasted fairly long in vivo (Chapter 5), and had minimal effects on chondrocyte
function and proliferation, similar to the findings of other researchers [6, 24, 26]. Since the dyes are long-lasting and non-toxic, and have been applied successfully in tracking implanted cells (Chapters 4 and 5), they will likely be useful in future \textit{in vivo} studies which are longer in duration than those presented in this dissertation.

Several future \textit{in vivo} studies are possible. Longer-term \textit{in vivo} studies are necessary in order to gain insight into the efficacy of tailored stratified cartilage tissues for repairing focal cartilage defects. Various endpoint analyses would be useful for the aforementioned \textit{in vivo} studies. First, determination of the recovery of implanted cells (proportion present at the time of retrieval compared to that present at the time of implant) may indicate mechanisms underlying repair quality at later time points. Preliminary studies (Appendix B) have indicated the possibility of determining a relationship with the degree of integration present between implant and host with proximity of implanted cells. In the future, it would be important to determine the degree of integration between implant and host (Appendix B) at various time points \textit{in vivo}.

Semi-quantitative histological analyses of tissues at the time of implant and retrieval (Chapters 4 and 5) provided information regarding phenotype of cells as well as the general quality of tissue. Extending these analyses further, to include molecules such as type X collagen and various glycosaminoglycans (biglycan, decorin, fibromodulin) would be useful. Quantitative collagen typing analysis would also further the understanding of the state of the construct at the time of implant and retrieval.

Functional assessment of the retrieved tissues at various time points conducted \textit{in situ} with indentation mechanical tests [1] would be useful. Additionally, following the progressive development and quality of repair of different types of constructs
(stratified versus mixed) in vivo, as well as the role of the host cell response (infiltration) on cartilage repair would be critical for eventual clinical application of stratified cartilage tissues.

From the studies presented in this dissertation, as well as others [2, 19, 21], it appears that host cell infiltration of the cartilage defect is prevalent in vivo particularly at early time points (Chapter 4). Determining the specific source of host cell infiltration (bone marrow derived cells versus cells derived from the synovial fluid) via radiolabeling, for example, would be useful in further understanding host-implant interactions. A structural membrane barrier [10] could be implemented as a means to prevent host cell infiltration and then determine the effects of absence of host cell infiltration on repair quality. Implantation of a stiffer, mechanically mature construct able to withstand compressive loading associated with the in vivo environment [16] could potentially enhance retention of implanted cells. Implantation of a cell-seeded biodegradable scaffold tailored to degrade greater than 1 week after implantation could potentially be useful in preventing early host cell infiltration.

6.4 Other Promising Tissue Engineering Strategies for Cartilage Repair

There are several promising tissue engineering strategies for articular cartilage repair of focal defects in addition to the approach described in this dissertation, of generating stratified cartilaginous tissues for implantation in vivo. Various cell sources, scaffolds, and implantation methods have been investigated.

The use of different cell sources and scaffolds for promoting chondrogenesis in vivo has been explored. Progenitor or stem cells from bone marrow [17], periosteum [7], fat [25, 28], as well as chondrocytes from various types of cartilage (articular, auricular, nasal) have been also used to generate engineered cartilage. Mesenchymal
stem cells [23] have also been recently combined with novel nanofibrous scaffolds which appear to support chondrogenesis [13, 14]. Previously, chondrocytes have been combined with biodegradable materials such as poly-lactic acid (PLA) and poly-glycolic acid (PGA) [8]. Other biocompatible materials include collagen [12], hyaluronan [18], and silk [27].

Injectable polymeric biomaterials which undergo rapid sol-gel transformation in situ are potentially useful as space-filling materials for repair of focal cartilage defects, and could have the added benefit of minimally invasive delivery by arthroscopic implantation. Chondrocytes or chondrogenic cells can likely be combined with polymer-peptide solutions and then can either be cultured in vitro [11] or injected and thermally triggered to covalently crosslink, solidify in vivo, and integrate with native cartilage tissue. In order to encourage integration of hydrogel materials with native cartilage in vivo, the use of an enzyme, transglutaminase has been explored. Specifically, biocompatible polyethylene glycol (PEG) conjugated polymer hydrogels formed by tissue transglutaminase crosslinking have been developed [4, 5, 9, 20]. Facilitation of integration by transglutaminase crosslinking is particularly motivated by the natural expression of tissue transglutaminase in many components of cartilage matrix (collagens II, III, V, XI; fibronectin) and the ability of these components to act as cross-linking substrates for the enzyme [22]. In addition, the interplay that occurs in vivo between physical stimuli and matrix molecules, as facilitated by mechanotransduction, will likely have an impact on the resulting biomechanical properties of the repair tissue, including integrative repair occurring between the repair tissue and native (host) cartilage.
6.6 keys to success in articular cartilage repair

The success of articular cartilage repair will depend on several aspects. One likely requirement is the presence of implanted cells, of the appropriate phenotype, which should be retained in sufficient number and in an organized fashion in the defect in order to fill the defect with newly synthesized extracellular matrix and to promote integration with the surrounding host tissues. Translation of in vitro generated constructs to in vivo applications is critical to determining an effective cartilage repair strategy. Determining the contribution of host-derived (synovial fibroblasts and bone marrow stromal cells) and implanted (superficial and middle cells) to repair, as well as cellular fates involved in cartilage repair in vivo (Figure 6.1), of articular cartilage defects has implications for current and future repair therapies. Controlling cell trafficking in vivo may be critical for successful cartilage repair. In addition, elucidating important implant characteristics for cartilage repair will impact current tissue engineering strategies, i.e. designing a cell-laden construct that is less penetrable by non-implanted cells, more likely to be retained in the implant region, and contains cells of the appropriate phenotype.
Figure 6.1: Cellular fates in cartilage repair.
6.6 References


A.1 Introduction

The efficacy of cell-laden tissue-engineered constructs for repairing articular cartilage defects is likely to depend on retention of the implanted cells. Cartilaginous tissues have been fabricated to have zone-specific features of native cartilage using chondrocytes isolated separately from the superficial (S) and middle (M) layers of articular cartilage [5, 6]. Such tissues have been implanted in vivo in the mini-pig in preliminary studies [1]. Since the duration for cartilage construct formation is lengthy, methods to stimulate construct growth would be useful. With chondrocytes isolated from full-thickness articular cartilage, the inclusion of bone morphogenetic protein-7 (BMP-7, or osteogenic protein-1, OP-1) in serum-supplemented medium stimulates proteoglycan and type II collagen synthesis [9, 10] and promotes formation of tissue-engineered cartilage [7]. The objectives of the present study were to determine if BMP-7 enhances matrix formation (1) in cultures of S and M chondrocytes in alginate, and also (2) in stratified cultures of cartilaginous tissues.
A.2 Methods

Chondrocyte Harvest and Expansion

Articular cartilage was harvested from the S (<0.2 mm depth) and M (0.4-1.0 mm) layers of the patellofemoral groove of adolescent Yucatan mini-pigs (4-6 m.o.), and used to isolate S and M chondrocyte subpopulations by sequential digestion in pronase and collagenase. Chondrocytes were plated in monolayer at low density (10,000 cells/cm²) and incubated in DMEM with 10% FBS and 25 μg/ml ascorbic acid until cells were 80% confluent. Cells were released with trypsin-EDTA, and some S and M cells were labeled with PKH26 (S*, M*) [2].

Effects of BMP-7 on CM and FRM in Alginate Beads

Chondrocytes were cultured in alginate beads [10] (n=2 knees) in DMEM/F12 with 20% FBS and 25 μg/ml ascorbic acid ±50 ng/ml rhBMP-7 (R&D). Cells were released from beads on days 1 and 14. The resulting suspension was centrifuged to separate the cells with associated matrix (CM), in the pellet, from the far-removed matrix (FRM), in the supernatant [7]. CM and FRM were solubilized with proteinase K. Portions of digests were analyzed to quantify the content of glycosaminoglycan (GAG) [4], collagen (COL) from hydroxyproline [12], and DNA [8].

Effects of BMP-7 on Construct Properties

In Vitro Fabrication.

S* and M cells (n=6 knees) were cultured in medium with BMP-7 in alginate beads for 14 days, and used to fabricate S*/M stratified cartilage constructs at a proportion of 25/75 with final density of 5 million cells/cm² cross-sectional area.³ Constructs were incubated in medium ±BMP-7 for 4 weeks.
Biomechanical Analysis.

The thickness of individual samples was measured at multiple locations using a contact-sensing instrument. A 9.6 mm diameter disk was isolated and tested to determine the equilibrium confined compression modulus [11].

Biochemical Analysis.

Portions of each construct were weighed wet and digested with proteinase K. Portions of the digest were analyzed for sulfated GAG [3], COL, and DNA.

Maintenance of Stratification In Vitro.

Portions of constructs were fixed in 2% paraformaldehyde, incubated with 0.01 mg/ml Hoechsht 33258 to counterstain cell nuclei, frozen, sectioned 30 μm in the vertical orientation, and imaged by fluorescence microscopy. The PKH26-labeled cells and H33258-labeled cell nuclei were localized by color-segmenting images and thresholding, and their densities determined as a function of depth in 10 equal-sized bins.

Statistical Analysis:

Effects of BMP-7, cell type, PKH26, and day were assessed by ANOVA with Tukey post-hoc tests. Repeated measures-ANOVA was used to assess the effects of BMP-7 treatment and depth. Data are expressed as mean±SEM.

A.3 Results

Effects of BMP-7 on CM and FRM in Alginate Beads

By day 14, DNA content of beads was affected by cell type (p<0.05) with an interactive effect of cell type and PKH26 (p<0.001), and no significant effect of BMP-
7 (p=0.06, Fig. A.1A). S* cultures had 64% more DNA than S cultures (p<0.001). M and M* cultures were not significantly different (p=0.07). CM-GAG/bead was affected by cell type (p<0.05) and BMP-7 (p<0.001) with interactive effects of cell type and PKH26 (p<0.01), and BMP-7 and PKH26 (p<0.05, Fig. A.1B). BMP-7 treated cells accumulated 91% more CM-GAG/bead than non-BMP-7 treated cells. COL was affected by cell type (p<0.05) and BMP-7 (p<0.001) but not PKH26 (p=0.97, Fig. A.1C). BMP-7 Effects of BMP-7 on Construct Properties

BMP-7 increased the thickness of constructs by 56% (p<0.001) and GAG content by 428% (p<0.05), without a significant effect (+58%, p=0.4) on COL content (Fig. A.2ABC). Constructs treated with BMP-7 were easier to handle than those without (Fig. A.2D), although the compressive modulus of constructs with (10±6 kPa) and without (6±2 kPa) BMP-7 were similar (p=0.87). Analysis of the cells in S*/M cartilaginous tissue showed PKH26-labeled cells to be concentrated in the superficial 20% of the construct (p<0.05 to 0.001 compared to deeper regions, Fig. A.3), with 86% of cells in this region being identified as PKH26-labeled. BMP-7 did not affect the depth-variation in PKH26-labeled cells (p=0.54), in overall cells (p=0.99), or in percentage of PKH26-labeled cells (p=0.64).
Figure A.1. Effects of cell type, BMP-7, and PKH26 labeling on (A) DNA, (B) GAG, and (C) COL content in the CM (■) and FRM (□) of cultures on day 14. n=2-3, (●) p<0.001, (♦) p<0.05.
Figure A.2. Effects of BMP-7 on construct properties. (A) Thickness. (B) GAG density. (C) COL density. (D) Gross morphology. n=6-9, (●) p<0.001, (♦) p<0.05.
Figure A.3. (A) Cellularity with depth for PKH26-tagged (■, ●) and total (□, ○) cells in S*/M constructs treated with (■, □) and without (●, ○) BMP-7. (B) Percentage of cells detected with PKH26. (C) Fluorescence micrographs of representative constructs. n=4-10, bar = 100 μm.
A.4 Discussion

These results indicate that addition of BMP-7 to culture medium, supplemented with 20% FBS, has a stimulatory effect on matrix synthesis by S and M chondrocytes, both in alginate bead and stratified cartilaginous tissue constructs. Since stratification was maintained in S*/M constructs with BMP-7 stimulation of both alginate and construct cultures, such constructs could be used to assess the role of S cells in the repair of articular cartilage defects. Long-term in vivo studies will be needed to assess the efficacy of such implants.
A.5 References


APPENDIX B

RELATIONSHIP OF CELLULARITY NEAR THE INTERFACE WITH DEGREE OF INTERFACE BONDING

B.1 Introduction

Cartilaginous tissues have been fabricated to have zone-specific features of native cartilage using chondrocytes isolated separately from the superficial (S) and middle (M) layers of articular cartilage [5, 6]. Such tissues have been implanted in vivo in the mini-pig in preliminary studies [1, 2] (Chapters 4 and 5). One important feature of repaired focal cartilage defects is the degree of integration between the implanted tissue and native host cartilage [12]. Integrative cartilage repair has been previously shown to depend on the presence of viable, metabolically active cells [7, 8, 11] as well as deposition and cross-linking of newly formed collagen [3, 4]. Adhesion of implanted cells or cell-laden construct to native host tissues is likely necessary for repair cells to assemble matrix both in the bulk of the defect as well as at or near the interface [9]. The degree of integration has been previously analyzed from histological images [10]. The objectives of the present study were to determine if cellularity near the defect tissue-host cartilage interface had a correlative relationship with the degree of interface bonding.
B.2 Methods

Sample preparation

Portions of tissues retrieved after implantation \textit{in vivo} (Chapter 5) were histologically analyzed. Samples were fixed in 4\% paraformaldehyde in PBS, embedded in paraffin, and sectioned vertically 5 \textmu m thick. Serial sections were stained with Safranin O/FastGreen (FG). Results were documented by brightfield microscopy using a Nikon Eclipse TE 300 microscope (AG Heinze, Irvine, CA) equipped with SPOT RT camera (Diagnostic Instruments, Burlingame, CA).

Determination of percentage of bonding at interface

The percentage of bonding at the interface between the repair tissue and adjacent host cartilage of the tissue was quantified by drawing line tracings using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) and measuring the lengths of these lines with ImageJ software (ImageJ, Bethesda, MD).

Determination of cellularity as a function of distance from the interface

The density (\#cells / mm$^2$) of host cells as well as cells in the defect region was quantified as a function of distance from the interface. Briefly, cells were manually identified using Adobe Photoshop. The articular surface, cartilage-bone interface, non-tissue regions (gaps or holes resulting from sectioning), and cartilage-defect interface were traced, digitized, and a best fit line to each of these landmarks was determined. The height of the articular cartilage was determined as the average distance between the surface and cartilage-bone interface. Bins measuring 10, 20, 50, or 100 \textmu m were created starting at the interface and continued for 300 \textmu m from the interface (in both directions, towards host tissue or defect tissue). The number of cells per bin area was
determined by dividing the number of cells by the tissue area, determined by subtracting non-tissue area from the total bin area (product of the bin size and height of cartilage in that bin).

Statistical Analysis

Pearson correlation was applied to determine dependencies of the percentage interface bonded with cellularity near the interface.

B.3 Results

Determination of percentage of bonding at interface

Native cartilage and repair tissues at \textbf{t=4 wk} showed significant differences in the percentage of bonded interface (Table 5.3, p<0.05) although the percentage of bonded interface was generally good, and similar, between tissues filling empty defects (93±5\%) and S*/M^\ (75±11\%) and S*+M^\ (54±28\%) (p=0.09 – 0.6) retrieved tissues.

Samples analyzed

Samples analyzed exhibited a range of histological features (Fig. B.1). For some samples, tissues filling the defect region extended over the adjacent host cartilage (Fig. B.1C, B.4). Additionally, tissues filling the defect region sometimes had an irregular surface (Fig. B.1B, B.3). Differences in matrix staining were also noted with some samples exhibiting positive glycosaminoglycan (GAG) staining (Fig. B.1A, B.2) and others negative staining (Fig. B.1AB, B.2). Examples of surface, bone, and interface tracings for the aforementioned conditions are show in Figures B.2, B.3, and B.4.
Determination of cellularity as a function of distance from the interface

Cellularity of tissues filling the defect region near the interface generally had a negative correlation with the percentage interface bonded (Fig. B.5). Cellularity of tissues filling the defect region small distances from the interface (10 and 20 μm) correlated negatively with the percentage interface bonded (10 μm: $R^2=0.66$, $p<0.01$; 20 μm: $R^2=0.56$, $p<0.05$). Cellularity of defect tissues larger distances from the interface (50 and 100 μm) showed a tendency towards a negative correlation with the percentage interface bonded ($p=0.2$). Cellularity of the adjacent host cartilage did not appear to have a correlative relationship with the percentage interface bonded ($p>0.4$).
Figure B.1. Examples of samples analyzed for cellularity and interface bonded. (A) Defects receiving $S^*/M^*$ tissues, (B) $S^*+M^*$ tissues, or (C) no treatment. Arrowheads indicate location of interface.
Figure B.2. Schematic of methods used to determine cellularity as a function of distance from the interface: (A) original SafO/FG image with cells manually identified and surface, bone, and interface tracings, (B) processed image with cells depicted as white dots and graded bins (100 μm) as a function of distance from the interface, (C) resulting cellularity as function of position relative to the interface (position 0). Arrowheads indicate location of interface.
Figure B.3. Schematic of methods used to determine cellularity as a function of distance from the interface: (A) original SaFo/FG image with cells manually identified and surface, bone, and interface tracings, (B) processed image with cells depicted as white dots and graded bins (100 μm) as a function of distance from the interface, (C) resulting cellularity as function of position relative to the interface (position 0). Arrowheads indicate location of interface.
Figure B.4. Schematic of methods used to determine cellularity as a function of distance from the interface: (A) original SafO/FG image with cells manually identified and surface, bone, and interface tracings, (B) processed image with cells depicted as white dots and graded bins (100 μm) as a function of distance from the interface, (C) resulting cellularity as function of position relative to the interface (position 0). Arrowheads indicate location of interface.
Table B.1. Defect and host cellularity values in the nearest bin to the interface for bins sized 10, 20, 50, and 100 μm.

<table>
<thead>
<tr>
<th>Bin size</th>
<th>10 μm</th>
<th>20 μm</th>
<th>50 μm</th>
<th>100 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Defect cellularity [10^4 cells/cm²]</td>
<td>Host cellularity [10^4 cells/cm²]</td>
<td>Defect cellularity [10^4 cells/cm²]</td>
<td>Host cellularity [10^4 cells/cm²]</td>
</tr>
<tr>
<td>Empty</td>
<td>0.03 ± 0.00</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.00</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>S^M^N</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>S^M^A</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.00</td>
</tr>
</tbody>
</table>
Figure B.5. Relationship between cellularity and interface bonded. (A, C, E, G) Defect and (B, D, F, H) host cellularity 10 μm (A, B), 20 μm (C, D), 50 μm (E, F), and 100 μm (G, H) distance from the interface. For defect treatment cellularity, $S^*/M^*$ (■), $S^*/M^*$ (▲), and tissues filling empty defects (♦) are shown.
B.4 Discussion

These results establish a methodology for quantitatively determining cellularity as a function of distance from a cartilage-defect interface. The distribution of cells in a range of samples analyzed was determined (Fig. B.1-B.4). All samples analyzed were fairly well bonded (>50% bonded). Cellularity of defect tissues larger distances from the interface (50 and 100 μm) had a tendency towards a negative correlation with the percentage interface bonded (Fig. B.5). Cellularity of the adjacent host cartilage did not appear to have a correlative relationship with the percentage interface bonded (Fig. B.5).

In the future, the method described here could be applied to determining the distribution of cells with respect to other interfaces e.g., cartilage-bone. A variation of this approach has been previously applied to localization of cells as a function of depth from the articular surface (Chapters 4 and 5) [1]. Longer durations in vivo may depict a clearer relationship between the presence of cells near the interface and the degree of bonded interface.

Interactions between host and implant at both the articular and subchondral surfaces may markedly affect cell retention and overall features of the cartilage repair process in vivo. These results suggest that, at an early timepoint in vivo (4 weeks), cellularity near the interface may not influence the percentage interface bonded. After 4 weeks in vivo, all defect treatments were bonded similarly at the interface between the defect region and adjacent host cartilage. Since the duration of 4 weeks in vivo is still early in the in vivo repair of focal cartilage defects, longer term in vivo studies
may reveal differences between defect treatments, in particular between cell-laden tissue implants and no treatment (empty defect).
B.5 References


